Extracellular matrix proteolysis by bronchoalveolar leukocytes in experimental pneumoconiosis

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I declare that this thesis was written by me
and that the work contained herein is my own.

Geraldine Marie Brown

"What we call the beginning is often the end

And to make an end is to make a beginning."

T.S. Elliot - Little Gidding

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ABSTRACT

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Silicosis and coalworkers' pneumoconiosis are lung diseases associated with occupational exposure to silica and coalmine dust, respectively. The definitive lesion in both diseases is lung fibrosis but emphysema accompanies coalworkers' pneumoconiosis and is also reported to be present in silicosis, although its relation to the fibrotic lesion is still controversial. A characteristic feature of pneumoconiosis is the accumulation of increased numbers of inflammatory leukocytes in the alveolar region of the lung and these cells have been implicated in the pathogenesis of the disease. Neutral proteinases, released by inflammatory leukocytes, have been shown previously to damage cellular and connective tissue components of the alveolar septa and may thus contribute to the tissue derangements of fibrosis and emphysema. A role for leukocyte neutral proteinases in the aetiology of the pneumoconioses emanates from studies demonstrating both increased levels of elastase in pneumoconiotic bronchoalveolar lavage fluid and increased elastase secretion by bronchoalveolar leukocytes from pneumoconiotic patients. However,there have been few studies to specifically investigate the proteolytic activity of the bronchoalveolar leukocytes in pneumoconiosis. The aim of the present study was, therefore, to assess the bronchoalveolar leukocyte response and proteolytic activity of the leukocytes in a rat model of pneumoconiosis.

An assay, based on the breakdown of [^{125}I]fibronectin, that would measure the overall proteolytic activity of the bronchoalveolar leukocytes and indicate their potential to damage the septal connective tissue, was developed and validated. Increased proteolytic activity was found in the inflammatory bronchoalveolar leukocyte populations and so the relative role of macrophages and neutrophils was assessed by separation into distinct populations. Neutrophils had the greatest ability to degrade connective tissue molecules, being at least 5-fold more active than resident macrophages but inflammatory macrophages also had increased proteolytic activity that was 3-fold more than the control macrophages.

The important features governing the inflammogenicity of particles were addressed by measuring the inflammation-generating properties of a variety of fibrogenic and non-fibrogenic particles in rats exposed by intratracheal instillation or inhalation. To assess the extent of the alveolitis generated by such treatment, the number and type of leukocytes recruited to the alveolar region was measured by bronchoalveolar lavage and the proteolytic activity of the leukocytes was assessed. Multiplying the total number of leukocytes recruited by the proteolytic activity per cell gave an indication of the potential proteinase burden in the alveolar region. In the acute phase following intratracheal

exposure, the fibrogenic dust quartz elicited levels of leukocyte recruitment that were similar to that evoked by non-fibrogenic particles and the proteolytic activity of the leukocytes was also similar. However, only quartz had the ability to maintain a sustained alveolitis in which the proteolytic activity of the bronchoalveolar leukocytes remained elevated. This indicated that the duration, rather than the initial magnitude of the inflammation might be an important factor in determining the pathogenic potential of dusts. This suggestion was supported by the finding that in rats exposed to coalmine dust by inhalation, the inflammatory response gradually increased with duration of dust exposure but it did not exceed the acute response to intratracheally injected particles. The inflammation did, however, persist long after the cessation of dusting and so the difference between fibrogenic and non-fibrogenic particles appears to be related to the duration of the inflammatory response which they can evoke in the lung. The sustained alveolitis with fibrogenic particles was not related to lack of clearance of dust from the lung. Both quartz and coalmine dust elicited a sustained alveolitis, but titanium dioxide, which is no more readily cleared than silica or coalmine dust, failed to sustain the inflammation.

Altering the surface of quartz particles by coating them with aluminium lactate reduced their ability to recruit inflammatory leukocytes but did not alter the proteolytic activity of the leukocytes. The tissue response to the coated quartz particles was also less than that elicited by native quartz with fewer and less-severe lesions.

The foregoing serve to substantiate the role of inflammatory leukocytes in the pathogenesis of the pneumoconiotic disease process. Only when there is a sustained alveolitis with an overall increased proteinase burden, does pathological change occur in the lung. Reducing the magnitude and/or the duration of the alveolitis markedly suppresses the development of the lesions.

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INTRODUCTION

1. INTRODUCTION

1.1. Aims of the thesis

Silicosis and coalworkers' pneumoconiosis are lung diseases associated with occupational exposure to silica and coalmine dust respectively. The definitive lesion in each case is lung fibrosis but this may also be accompanied by emphysema. Chronic alveolitis is a characteristic feature of both diseases and the inflammatory leukocytes are deemed to be central to the pathogenic process. Degradation of lung tissue by leukocyte proteinases has been implicated in the development of lung pathology in other chronic inflammatory diseases of the lung such as adult respiratory distress syndrome (ARDS) and idiopathic pulmonary fibrosis (IPF). By inference, the same may also be true of coalworkers' pneumoconiosis and silicosis but little previous work has specifically addressed this question. The primary aim of the work described in this thesis was, therefore, to investigate the role of connective tissue proteolysis by inflammatory leukocytes in the pathogenesis of coalworkers' pneumoconiosis, silicosis and emphysema. The specific aims of the thesis are described in the following sections.

1.1.1. Development of the proteolysis assay

Much of the previous work on leukocyte proteinases has investigated the proteolytic activity of leukocytes by measuring their secretion or content of a specific proteinase. A primary objective of this thesis was to develop an assay of connective tissue proteolysis which would measure, the entire activity of viable bronchoalveolar leukocytes in degrading connective tissue molecules in vitro.

1.1.2. Assessment of the relative proteolytic activity of inflammatory macrophages and neutrophils

The leukocytes of a non-immune alveolitis are comprised mainly of macrophages and neutrophils. Both types of leukocyte secrete proteolytic enzymes and could therefore contribute to tissue damage in chronic alveolitis. Knowing the potential contribution that each leukocyte type could make to the overall proteinase burden in the lung might be of use in developing therapeutic intervention in the disease process. A further aim of this thesis was therefore to obtain inflammatory bronchoalveolar macrophages and neutrophils and to assess the relative proteolytic activity of each population.

1.1.3. Investigation of the role of persistent inflammation in causing tissue injury

Exposure to harmful mineral dust particles is frequently associated with pathological change in the lung but most microbial infections resolve without pathological change. The difference between these two types of inflammogen in terms of their ability to cause long-term damage in the lung may be related to the degradeability of the microbes. A further aim of the thesis was therefore a) to investigate differences between microbial and mineral dust challenge in the lung by measuring the persistence of alveolitis following deposition of degradeable and non-degradeable particles by intratracheal injection, and b) to assess the ability of bronchoalveolar leukocytes from the lungs of rats injected with microbes or mineral dust particles to degrade connective tissue molecules using the proteolysis assay described in 1.1.1. above.

1.1.4. Assessment of the alveolitis in rats exposed to coalmine dust by inhalation

Intratracheal injection is a valuable tool in answering basic questions regarding the pathogenesis of lung disease but it is not entirely representative of the real life situation when dust is inhaled. Deposition of airborne dust in the alveolar region is related to aerodynamic properties of the dust in the complex branching airway system of the lungs. An additional aim of the thesis was therefore to assess the development of alveolitis in rat lungs exposed to coalmine dust by inhalation and to assess the proteolytic activity of the bronchoalveolar leukocytes as above.

1.1.5. Investigation of the biological reactivity of quartz in relation to the surface of the quartz particles

Some forms of silica, especially quartz, can be highly fibrogenic and the pathogenicity of some coalmine dusts has been related to their quartz content. The harmful effects of quartz are thought to be associated with silanol groups on the particle surface which may react with cells by acting as hydrogen donors and thus cause membrane damage. The final aim of the thesis was to investigate the importance of the quartz surface to its biological reactivity by measuring the ability of surface-modified quartz to elicit and sustain an alveolitis.

1.2. Origin and functions of leukocytes

Leukocytes, or white blood cells, are a major component of the host defence system of the body (Klempner, Styrt and Ho, 1989). They originate in the bone marrow and, in subsequent divisions, differentiate to form a variety of lineages with different functional capacities.

They then enter the bloodstream where they remain for varying periods of time before passing into the tissues, prior to further differentiation. Pluripotent stem cells give rise to two distinct lineages:- (1) lymphoid - which differentiates to form the T- and B lymphocytes and (2) myeloid- which gives rise to the monocyte/macrophages, the polymorphonuclear leukocytes (neutrophils, eosinophils and basophils) and also to platelets and mast cells (Nauseef, 1989). Each of these cell types has a specific role to play in host defence.

Monocytes form a pool of cells (2-20% of circulating leukocytes) in the bloodstream, which migrate into the tissues to give rise to the more-differentiated tissue macrophages. Macrophages and monocytes share similar functional characteristics such as phagocytosis and killing of bacteria, antigen presentation to T lymphocytes and secretion of regulatory molecules (cytokines/monokines) (Vaes, 1985; Ho, 1989). Via the latter two functions, macrophages can orchestrate the responses of other leukocytes and are thus pivotal to the host defence system (Vaes, 1985).

Amongst the polymorphonuclear leukocytes, neutrophils are the most common, forming 40-75% of circulating leukocytes in the bloodstream; eosinophils and basophils represent less than 0.2%. The primary function of the neutrophil is bacterial killing and this is reflected in the rapid recruitment of large numbers of neutrophils to sites of infection/inflammation (Baggiolini and Dewald, 1985).

Lymphocytes form 20-50% of the circulating leukocytes, and are also found in lymphoid organs in the tissues. Both T- and B lymphocytes are involved in specific recognition of antigen and the response to it. T cells responding to antigen challenge undergo clonal expansion and then perform several different functions including "help" to other lymphocyte responses (T_h cells), cell-mediated killing (T_c cells) and production of regulatory lymphokines (T_{dth} cells); gamma-interferon is a particularly potent mediator secreted by T_{dth} lymphocytes and is a very effective activator of macrophages and other cells. B cells secrete antibodies, specific globular proteins which combine with antigen; this interaction neutralises microbes, enhances phagocytosis, activates complement and facilitates antibody-dependent cellular cytotoxicity.

1.3. The host defence systems of the body

The responses of the host defence system can be divided into two categories - innate and adaptive (Roitt et al, 1985). The body is continually exposed to a low level of microbial challenge, particularly at epithelial surfaces such as those lining the respiratory tract, the digestive tract and the skin. This low-level threat is dealt with by the innate immune

system which comprises:- (a) soluble factors such as lysosyme and complement (activated directly by microbes via the alternative pathway) which act to kill or opsonise the microbes; (b) cellular elements- these are the NK cells which kill virally infected cells, and macrophages and neutrophils which phagocytose and then kill microbes. Microbial killing by macrophages and neutrophils can take place both intra- and extracellularly by release of proteolytic enzymes and reactive oxygen species into the extracellular environment.

Low level bacterial challenge can be dealt with by the tissue macrophages and NK cells but where the microbial challenge increases to levels that overload these first line defences (infection), the mechanisms of innate immunity are enhanced and inflammation results. At the site of inflammation, increased capillary permeability permits diffusion of important defensive plasma components such as immunoglobulins to the site of tissue injury (Jones et al, 1982). The acute phase proteins, induced in the liver by increased circulating interleukin-1 (IL-1, released by leukocytes at the focus of inflammation) enhance complement activation to generate the anaphalatoxin, C5a. C5a is also generated following activation of complement by the alternative pathway through interaction with bacterial surfaces. The generation of a C5a gradient at the site of inflammation then causes blood leukocytes (first polymorphonuclear leukocytes and then monocytes) to migrate from the blood capillaries up the chemotactic gradient, and into the inflamed tissue. Other mechanisms involved in leukocyte recruitment are discussed in further detail in 1.5.3. Neutrophils constitutively contain more proteolytic enzymes than macrophages and the initial influx of neutrophils to the inflammatory site generally facilitates rapid clearance of the invading organism. In this respect neutrophils are generally considered to be the classical cell of the acute inflammatory response. Monocytes are recruited later than neutrophils and may therefore be of greater importance to the regenerative phase of the inflammatory response (Vaes, 1985; Fotev et al, 1987).

The adaptive immune response involves macrophages and T- and B lymphocytes principally (Roitt et al,1988). During initial exposure to immunogenic material, limited B- and T cell responses are produced (see 1.2). In addition, a class of lymphocytes called memory cells are generated. On subsequent exposure to the antigen, this enables a quicker and larger response to occur through clonal expansion of the memory cells. Macrophages are central to the adaptive immune response through their ability to digest phagocytosed microbes and then present the bacterial antigen, to the immune system at their cell surface. Adaptive immune responses go on at a constant low level in the body without inflammation developing but when the amount of immunogenic material in the body becomes increased,

high levels of circulating antibody and/or effector T cells are produced and an inflammatory response develops as above.

The two systems of innate and adaptive immunity are thus closely interlinked at all levels and involve interactions between myeloid and lymphoid cells. The outcome of this cellular cooperation is the rapid elimination of microbes and protection against host tissue damage.

Leukocytes are found in all tissues of the body in anticipation of a microbial challenge. This is particularly true of the lung where exposure to inhaled material poses a constant potential threat of infection. This thesis is concerned particularly with macrophages and neutrophils obtained by bronchoalveolar lavage and so the specific role of these cells in lung defence and disease will be discussed in further detail in succeeding sections.

1.4. Host defence in normal lungs

1.4.1. Physical parameters

The air we breathe contains many particles but not all of these gain access to the delicate tissues of the alveolar region. There are several defence mechanisms in the upper respiratory tract which ensure that the majority of inhaled particles are cleared before they reach the deep lung (Raabe, 1984):- (a) nasal hairs- these act as filters to trap large particles; (b) the mucociliary escalator- the majority of particles which penetrate into the upper respiratory tract deposit on the walls of the bronchi and are trapped in the rafts of mucus produced by other cells, which line the whole of this area. Ciliated cells on the surface of the bronchi then waft the mucus, with trapped particles, up to the throat where it is eliminated from the lung by swallowing or spitting. The deposition process is aided by the branching structure of the bronchi. This forms a tortuous route for the airflow and so a large proportion of the particles impact at bronchial bifurcations; (c) deposition of inhaled material in the lung is also highly dependent on particle size - particles of large aerodynamic dynameter (>10 microns) deposit in the airways very quickly by impaction or sedimentation and are then removed on the mucociliary escalator, whilst some particles (<0.5 microns) tend to remain airborne and are carried out of the lungs in the expired air. Particles 1-5 microns in diameter penetrate to the alveolar region and have a high likelihood of depositing there (the alveolar deposition fraction).

1.4.2. Cellular aspects of host defense in normal lungs

The mucociliary escalator does not extend beyond the respiratory bronchioles and so for those particles which do penetrate to the alveolar region and deposit in the alveoli, some other clearance processes must operate. The resident macrophages are the primary line of

defence against inhaled particulate material in the alveolar region (Green et al, 1977) and are central to all host defence functions in that area. Most of the particles which deposit in the deep lung are phagocytosed by macrophages and then broken down within lysosomes or they may be transported intracellularly to the mucociliary escalator or to the lymph nodes (Lehnert et al, 1988).

The major means of removing microbial particles from the lung is killing and digestion by macrophages and neutrophils and this is mediated largely by two mechanisms:-

1 oxygen-dependent processes - reactive oxygen species such as superoxide anion, hydrogen peroxide and hypohalide are generated from molecular oxygen by oxidases. These oxidants are highly effective bactericidal agents (Klebanoff and Hamon, 1975; Babior, 1978). Killing via oxidative interactions with the bacterial membrane takes place in both the intra- and extracellular environments (Nathan et al, 1980);

2 oxygen-independent processes - shortly after phagocytosis of microbes the phagosome fuses with an enzyme-containing lysosome and the pH of the phagolysosome then falls rapidly to around pH 4.0. The low pH and the presence of cationic proteins is thought to contribute to microbial killing (Ohlsson et al, 1978). Digestion of the killed microbes is mediated by the lysosomal proteinases which can operate both intra- and extracellularly. Both acid and neutral proteinases can contribute to the digestive process and are released by leukocytes in response to a phagocytic stimulus (Leoni and Dean, 1983; Henson and Johnston, 1987). However, digestion and elimination of microbes takes place largely intracellularly.

As previously described (1.3.), alveolar macrophages can also interact with lymphocytes to trigger the adaptive immune response. In the alveolus, macrophages phagocytose the invading pathogen and if the microbial insult is sufficiently intense, they may then migrate to the lymph nodes where they are involved in antigen presentation to T lymphocytes. This leads to clonal expansion of T- and B lymphocytes specific for antigens of the invading pathogen, with associated cell-mediated cytotoxicity by T lymphocytes and production of specific antibodies by B lymphocytes (Kaltreider, 1976).

1.4.3. Cellular profile of bronchoalveolar lavage leukocytes

Since its inception in the early 1970s, diagnostic bronchoalveolar lavage has been used extensively in the detection and monitoring of disease states both clinically (Reynolds, 1987) and experimentally (Dethloff et al, 1987). The technique permits sampling of the cellular and biochemical constituents present in the airspaces of the lung. The

bronchoalveolar leukocytes are considered to be representative of the interstitial response since the cellular profile of the bronchoalveolar cells is similar to those in the interstitium in biopsy tissue (Hunninghake et al, 1979; Haslam et al, 1980: Nibbering et al, 1989). The total number and differential count of the bronchoalveolar leukocytes are parameters which yield vital information in the diagnosis and staging of inflammatory lung disease (Reynolds,1987; Crystal et al,1981). The differential leukocyte count in normal bronchoalveolar lavage varies from species to species but the predominant leukocyte type is always the macrophage (Table 1).

Table 1. *Proportions of leukocytes in normal bronchoalveolar lavage in different species.*

		Differential count (%)		
Species	**Reference**	**macrophages**	**%neutrophils**	**%lymphocytes**
Human	(1)	85	<1	7-12
Rat	(2)	99	<1	<1
Monkeys	(3)	91	4	5
Sheep	(4)	84	2	14
Rabbits	(5)	98	1	1

(1) Reynolds, (1987), (2) Donaldson et al, (1988a), (3) Idell et al, (1987), (4) Glauser et al, (1988), (5) Shen et al, (1988)

1.4.4. Protection against injury to the host tissue

During breathing, the lung is continuously exposed to exogenous material. In order to prevent excessive response to the constant tissue insult, the normal function of alveolar macrophages is suppressive of immune and inflammatory responses (Holt, 1986) and resident alveolar macrophages in normal lungs are not activated. Although one of the functions of macrophages at other sites in the body is to present antigen to T lymphocytes and so activate them, in vitro studies have shown that resident alveolar macrophages reduce splenocyte proliferation (Kusaka et al, in press) and NK cell activity (Weissman et al, 1986). When alveolar macrophages do become activated (e.g. by the process of phagocytosis and killing of large numbers of microbes in the lung), the consequent release of proteolytic enzymes and oxidants into the extracellular environment is potentially hazardous to the host tissue (see 1.6.1 for further details).

Under normal circumstances "bystander injury" to the surrounding host tissue is prevented, or substantially restricted, by protective mechanisms which limit the activity of the released proteinases and oxidants. Antioxidant defences include catalase, a scavenger of hydrogen peroxide, superoxide dismutase which inactivates superoxide anion, glutathione

reductase and ceruloplasmin (Forman and Fisher, 1981; Aebi, 1984; Cantin and Crystal, 1985). Protection against proteolytic injury in the lung is thought to be mediated by a cascade of antiproteinases (Morrison, 1987), the most prevalent of which is α1-proteinase inhibitor (α1-PI), the major inhibitor of neutrophil elastase but not of macrophage enzymes. α2-macroglobulin (α2M) inhibits both macrophage and neutrophil proteinases (Travis and Salvesen, 1983), but it is a molecule of large molecular weight and is found in very small amounts in normal alveoli. However, the alveolar concentration of α2M is increased in lung inflammation due to both transudation from plasma and local production by macrophages (Nathan, 1987). Other proteinase inhibitors in the lung include inter α-trypsin inhibitor (Hochstrasser et al, 1972), α1-antichymotrypsin (Ohlsson, 1978) and antileukoproteinase (Kramps et al, 1988). Alveolar macrophages are involved in antiproteinase defence through their ability to secrete α1-PI (White et al, 1981a; Mornex et al, 1986) and α2M (Hovi et al, 1977; White et al, 1981b).

1.5. Bronchoalveolar lavage leukocytes in inflamed lung

1.5.1. Cellular profile of the lavage leukocytes

The proportion and total number of the various leukocyte types in human bronchoalveolar lavage are altered when inflammatory disease is present and this varies with the type and stage of disease (Crystal et al, 1981; Rossi, 1986). In patients with active sarcoidosis, the percentage of T lymphocytes may increase to around 40% (Thomas and Hunninghake, 1987), whilst in pneumonia there may be 70% neutrophils (Begin et al, 1986a). Other chronic inflammatory lung diseases also show marked increases in the number and proportion of neutrophils in bronchoalveolar lavage. In patients with rheumatoid lung and cryptogenic fibrosing alveolitis and also in silicotics and asbestotics (Robinson et al, 1988), the increase in neutrophils is accompanied by the appearance of eosinophils; in each case the total number of leukocytes is also markedly increased. Experimental models of lung disease have also revealed changes in the leukocyte profile of the lavage in rats (Morgan et al, 1980; Dethloff et al, 1987 ; Donaldson et al, 1988a), rabbits (Shen et al, 1988), monkeys (Idell et al, 1987), sheep (Begin et al, 1983) and hamsters (Verghese et al, 1988), the most common response being increases in the total number of inflammatory leukocytes and the proportion of neutrophils in the bronchoalveolar lavage.

1.5.2. Functional properties of bronchoalveolar lavage leukocytes

In addition to assessing changes in the cellular profile of bronchoalveolar leukocytes in disease states, the functional properties of the leukocytes are frequently assessed and are generally considered to be representative of the leukocytes of the entire lung parenchyma.

Functional differences between interstitial and bronchoalveolar leukocytes do exist but there are also many comparable features. Rat alveolar macrophages have been shown to have increased IgG-Fc receptor expression and to exhibit enhanced ability to inhibit splenocyte proliferation compared with interstitial macrophages (Holt et al, 1982). Weissler et al (1986) reported that human bronchoalveolar macrophages were less effective at stimulating a mixed lymphocyte reaction than macrophages from minced lung but were of similar activity in interleukin 1 production and ability to present antigen. The bronchoalveolar and interstitial populations were similar in expression of HLA-DR and were of comparable maturity as determined by staining with the differentiation antigen, MO2. Nibbering et al (1989), using a panel of monoclonal antibodies, showed differences in expression of some antigens between bronchoalveolar and interstitial macrophages in mice infected with BCG but fewer differences in control mice.

Assessment of the functional properties of bronchoalveolar leukocytes has been used to gain valuable information about the role of inflammatory leukocytes in lung disease, both clinically and experimentally. Human bronchoalveolar macrophages in lung disease show conflicting activities in the hands of different groups. They secrete increased IL-1 (Eden and Turino, 1986), spontaneously secrete more alveolar macrophage-derived growth factor (Bitterman et al, 1983) than control bronchoalveolar macrophages and yet have greater ability to suppress autologous lymphocyte proliferation (Fireman et al, 1988). Experimentally, altered bronchoalveolar leukocyte function has been expressed as reduced spreading and chemotaxis in guinea pigs injected intratracheally with silica (Dauber et al, 1982), decreased production of leukotrienes following smoke exposure in rats (Mobley et al, 1987) and increased ability to cause epithelial injury following exposure to a variety of mineral dusts (Donaldson et al, 1988b). Intratracheal injection of silica in rats leads to impaired chemotactic responses of the bronchoalveolar leukocytes (Donaldson et al, 1989) and increased IL-1 secretion (Kusaka et al, in press).

1.5.3. Mechanisms of leukocyte recruitment

Small amounts of inhaled particles, including microbes, are constantly deposited in the lungs. In the deep lung, this is largely dealt with by non-specific mechanisms involving the alveolar macrophages, as previously described (1.4.2), but when the burden of extrinsic material in the lung is excessive, leukocyte recruitment occurs and this results in inflammation in the alveolar region (Donaldson et al, 1988a). The inflammatory response is typified by increased alveolar epithelial permeability (Jones et al, 1982) and by increases in the total number of macrophages and in the number and percentage of neutrophils in bronchoalveolar lavage (Keogh and Crystal, 1982; Donaldson et al, 1988a).

The increase in leukocytes is thought to arise largely by recruitment from the bloodstream (Bowden et al, 1969) although local proliferation of macrophages may also account for some of the increase (Tarling et al, 1987). Leukocyte recruitment occurs in response to a chemotactic gradient such as that generated by microbial activation of complement (via the classical or the alternative pathways), where C5a is the principal chemotactic agent. Mineral dusts can also effect complement activation (Warheit et al, 1988) but to a much lesser extent than microbes. Complement activation by mineral dusts may be the means whereby the resident alveolar macrophages initially migrate within the alveoli to the site of dust deposition. It is unlikely, however, that this is the main cause of leukocyte recruitment in dust-inflamed lung since the degree of complement activation (measured by generation of leukocyte chemotactic activity in serum) engendered by dusts *in vitro*, does not correlate with their inflammogenic or pathogenic potential *in vivo* (K. Donaldson, unpublished observation).

An additional source of chemotactic activity is from lung cells themselves and chemotaxins are produced by activated macrophages (Herscowitz, 1985), neutrophils (Spilberg and Mehta, 1979) and epithelial cells (Chauncey et al, 1988). Macrophages exposed to a particulate stimulus *in vitro* release increased amounts of leukocyte chemotaxin (Lugano et al, 1981), and bronchoalveolar macrophages from humans or animals with active lung inflammation spontaneously secrete increased amounts of leukocyte chemotaxins *in vitro* (Hunninghake et al, 1980; Wesselius et al, 1984; Schoenberger et al, 1982; Verghese et al, 1988). Following the initial burst of inflammation, other sources of leukocyte chemotaxins may be generated if tissue injury occurs; dead cells (Privalova et al, 1980) and whole molecules or breakdown products of the connective tissue components collagen, (Postlethwaite et al, 1976), elastin (Hunninghake et al, 1981a; Senior et al, 1984), laminin (Terranova et al, 1986) and fibronectin (Norris et al, 1982) are chemotactic for leukocytes *in vitro*.

IL-1 and tumour necrosis factor (TNF) can enhance leukocyte recruitment to inflammatory sites by upregulating adhesion molecules on the capillary endothelium (Pohlman et al, 1986) or attachment molecules on leukocytes (Hogg, 1989). This enhances margination of leukocytes in post-capillary venules which is considered to be the first step in diapedesis of the leukocytes from the bloodstream into the inflamed tissue (Malech, 1988). In cigarette smokers, where there are increased numbers of leukocytes in the alveolar space, smoking leads to an increase in the transit time for neutrophils passing through the pulmonary capillaries (MacNee et al, 1989a). This could be taken as a general model for the micro-circulatory events occurring during inflammation due to other agents where the increased contact time between leukocytes and the capillary endothelium favours

diapedesis and emigration. The physiological basis for the increased transit time has not yet been fully revealed but decreased deformability of leukocytes, caused by cigarette smoke or inflammatory mediators such as chemotaxins, could cause them to take longer to negotiate the narrow alveolar capillaries (MacNee et al, 1989b). Decreased transit time of leukocytes in dust-exposed lung has not, as yet, been demonstrated.

1.5.4. Macrophage activation in inflamed lungs

Macrophage activation is manifest as complex alterations in the structure, metabolism and secretory status of these cells (Schneider and Dy, 1985; Johnston et al, 1986). Activated alveolar macrophages show increased spreading on glass and release increased amounts of oxidants, proteinases and cytokines; the latter can influence the functional status of other cell types in the alveolus. Macrophage activation can be instigated by a variety of stimuli, many of which are present in inflamed lung. Connective tissue components such as fibronectin and chondroitin sulphates are released by proteolysis of the extracellular matrix and are also secreted by activated macrophages (Rennard et al, 1981; Levitt and Ho, 1983). This can, in turn, cause macrophage activation:- elastin peptides increase cell movement and induce both oxidant and proteinase secretion in human monocytes (Fulop et al, 1986). Collagen stimulates production of prostaglandins and a monocyte stimulating factor in human monocytes (Dayer et al, 1982), acid hydrolase secretion and cytotoxicity to tumour cells in resident mouse peritoneal macrophages (Schorlemmer et al, 1980) and prostaglandin E2 and IL-1 production (Dayer et al, 1986) by human monocytes / macrophages. Fibronectin enhances oxidant production in human monocytes (Kuroiwa et al, 1988) and laminin enhances the phagocytic activity of human monocyte/macrophages (Bohnsack et al, 1985). Neutrophils can enhance macrophage function through secretion of IL-1 (Kusaka et al, in press) or a phagocytosis stimulating factor (Ishibashi and Yamashita, 1987). Chemotaxins such as C5a (Banks et al, 1988), various phagocytic stimuli (Schnyder and Baggiolini, 1978) and cytokines such as interferon gamma (Schultz, 1980), TNF (Beutler and Cerami, 1988), granulocyte macrophage colony stimulating factor (Metcalf, 1985) and IL-1 Martin and Resch, 1988), elicit macrophage activation *in vitro* and, in addition, many are secreted by activated macrophages (Dinarello, 1984; Metcalf, 1985; Nathan, 1987; Beutler and Cerami, 1988) thus a likely role for autoregulation of macrophages in inflamed lung can be envisaged. The effects of such cytokines can be synergistic (Billingham, 1987) and so, in inflamed lung where multiple inflammogenic stimuli, including cytokines may be present, the potential for macrophage activation to occur is likely to be great.

Alveolar macrophage activation has been demonstrated by various criteria in several disease states; these criteria include expression of activation antigens (Agostini et al, 1987) and enhanced uptake of ^{67}Gallium (Hunninghake et al, 1981b) in sarcoidosis and hypersensitivity pneumonitis. Bronchoalveolar macrophages obtained from sarcoid patients show increased secretion of IL-1 (Hunninghake, 1984) and gamma-interferon (Robinson et al, 1985) *in vitro*. In the interstitial lung diseases there is evidence of increased secretion of the alveolar macrophage-derived growth factor (Bitterman et al, 1983), fibronectin (Rennard et al, 1981) and platelet derived growth factor (Martinet et al, 1987). Alveolar macrophage activation has also been demonstrated experimentally as increased spreading (Donaldson et al, 1984) and enhanced IL-1 secretion in rats exposed to silica and asbestos (Oghiso and Kubota, 1986; Kusaka et al, in press). Increased oxidant production has been described in hamsters similarly exposed to pathogenic dust (Hansen and Mossman, 1987); chemotaxin secretion has been found in hamsters exposed to bleomycin (Schoenberger et al, 1982) and arachidonic acid metabolism is increased in macrophages from a rat model of lung granuloma (Kunkel and Chensue, 1983). In the milieu of inflamed lungs, activated macrophages are likely, therefore, to play a major part in orchestrating the development of the alveolitis.

1.5.5. Polymorphonuclear leukocytes in inflamed lung

The polymorphonuclear leukocytes are comprised of neutrophils, eosinophils, basophils and mast cells; of these, the neutrophils are the body's main cellular defence against invading microbes (Baggiolini and Dewald, 1985). Neutrophils are recruited to sites of inflammation in response to chemotactic stimuli (see 1.5.3.). Once in the tissues they undergo a process of activation, which is similar to that previously described for macrophages (section 1.5.4.) and is mediated by chemotactic (Showell et al, 1976; Snyderman and Goetzl, 1981) and phagocytic stimuli (Root and Metcalf, 1977; Baggiolini et al, 1980), by inflammatory mediators (Shaw et al, 1981; Snyderman and Goetzl, 1981) and by connective tissue molecules such as laminin (Terranova et al, 1986).

The process of activation triggers the release of various toxic products and inflammatory mediators by the neutrophils. The toxic products are involved in microbial killing, a violent process which can take place in the intra- or the extra cellular environments (see 1.4.2.). Neutrophils eliminate bacteria largely by means of two types of toxic product - oxidants and proteinases (Baggiolini and Dewald, 1985). These have the capacity to damage components of the host tissue and extracellular release of neutrophil oxidants (Fantone and Ward, 1985) and proteinases (Reiser and Last, 1986) is considered to be a major source of tissue injury in inflamed lung. Inflammatory mediators released by the

neutrophils include products of the cyclo-oxygenase (Goldstein et al, 1978) and lipoxygenase pathways (Smith et al, 1987), platelet activating factor (Betz and Henson, 1980) and IL-1 (Kusaka et al, in press; Tiku et al, 1986); all of these can further enhance the inflammatory response by recruitment and activation of inflammatory macrophages and neutrophils.

Eosinophils, basophils and mast cells are less commonly seen than neutrophils in bronchoalveolar lavage from inflamed lungs but they do occur in some diseases. Cationic proteins derived from activated eosinophils have been implicated in lung damage in ARDS (Hallgren et al, 1987) and eosinophils have also been implicated in the pathogenesis of pulmonary fibrosis (Peterson et al, 1987). Activation of basophils and mast cells by IgE, with consequent release of inflammatory mediators is thought to play a part in immunological diseases of the lung such as asthma and hypersensitivity pneumonitis (Marone, 1985) but little is known of their role in non-immunological lung disease.

1.5.6. Lymphocyte populations in inflamed lungs

Alterations in the lavage profile of lymphocytes or lymphocyte subsets in pneumoconiosis and bleomycin-exposed lung (Costabel et al, 1987; White et al, 1987) suggest that these cells may be involved in the pathogenesis of lung disease where an immune component is not immediately obvious. Lymphocytes, particularly T lymphocytes, may be involved in lung pathology through secretion of gamma-interferon. This cytokine is a very efficient activator of macrophages and can act synergistically with other cytokines such as IL-1 to modulate the inflammatory response. Stimulation of lymphocytes to secrete gamma-interferon occurs in response to antigen presentation in infected lungs. However, in a non-antigenic inflammation, such as that produced by mineral dust exposure, the initiating event for stimulation of lymphocytes may be secretion of IL-1 by activated macrophages (Oghiso and Kubota, 1986) and neutrophils (Kusaka et al, in press).

1.6. Mechanisms of leukocyte-mediated injury in inflamed lung

All tissue cells depend on the extracellular matrix for physical support and to maintain the microenvironment and structural integrity of the tissue. As in other tissues, the cells and extracellular connective tissue of the lung parenchyma form a dynamic matrix that is in a constant state of flux; both the cellular elements and the connective tissue are continually degraded and renewed as part of the normal "housekeeping" of the lung (Rennard and Crystal,1982). This constant renewal and replacement of the various components of the lung parenchyma ensures their integrity and is thus vital in maintaining the structure and function of the entire organ. In normal lungs, complex interactions between

the leukocyte populations, the fixed cells (i.e. the epithelial, endothelial and mesenchymal cells) and the connective tissue molecules of the lung parenchyma regulate the metabolism and replication of the cells and this, in turn, governs the amount of extracellular matrix that is deposited during normal connective tissue turnover and repair of minor lung injury. In inflamed lungs, the release of toxic products by activated macrophages and neutrophils causes injury to the delicate tissue of the alveolar septa and consequent imbalance in these regulatory interactions. If the injury persists and the connective tissue derangements become severe, this can result in permanently altered tissue structure and function. Leukocytes also have the capacity to stimulate proliferation of the fixed lung cells and to induce them to secrete connective tissue molecules. This has beneficial consequences for the lung in repairing damaged tissue by restoring normal tissue architecture. However, in pathologic conditions this type of stimulation may lead to excessive proliferation of fibroblasts and scar tissue formation due to deposition of excessive amounts or inappropriate types of connective tissue molecules.

1.6.1. Toxic products of inflammatory leukocytes

Inflammatory leukocytes release toxic products at sites of inflammation (see 1.4.2.) and these have the capacity to cause "bystander injury" to the surrounding host tissue. The most common types of injury are mediated by leukocyte proteinases and oxidants which can injure both the fixed cells and connective tissue of the alveolar septa.

1.6.1.1. Leukocyte-derived oxidants.

In vitro studies have demonstrated that leukocyte-derived oxidants can mediate injury to epithelial cells (Sugahara et al, 1986; Cantin et al, 1987) and endothelial cells (Harlan et al, 1985; Martin and Kachel, 1985) and can also damage connective tissue molecules (Audley and Chakrabarti, 1983; Monboisse et al, 1983; Riley and Kerr, 1985). Support for the proposal that these toxic leukocyte products are important in the pathogenesis of chronic inflammatory lung disease is found as increased oxidant production by bronchoalveolar leukocytes in coalworkers with progressive massive fibrosis (Wallaert et al, 1987) and in cigarette smokers (Hubbard et al, 1987). Confirmation that oxidant injury leads to fibrosis comes from animal experiments where oxidant gases such as ozone cause pulmonary fibrosis (Barr et al, 1988).

1.6.1.2. Leukocyte-derived proteinases.

Acid and neutral proteinases have also been implicated in the tissue injury associated with inflammatory lung disease. Acid proteinases are released in large amounts to the extracellular environment by inflammatory neutrophils and are found in increased amounts

in bronchoalveolar lavage fluid from inflamed lungs (Donaldson et al, 1988c). Although the extracellular milieu of the lung is usually maintained at neutral pH, local accumulations of activated neutrophils may acidify the surrounding tissue by release of granule contents and so permit the acid proteinases to act extracellularly (Davies and Bonney, 1980).

Leukocyte neutral proteinases are currently thought to be the major source of tissue proteolysis in inflamed lung. They are released in increased amounts by inflammatory leukocytes *in vitro* (see 1.7.3-1.7.5) and are also increased in the bronchoalveolar lavage in chronic inflammatory lung disease (see 1.7.7.). They have been shown to cause connective tissue damage *in vivo* (Janoff, 1985; Snider et al, 1986) and *in vitro* (McDonald et al, 1979; Werb et al, 1980) and also to mediate epithelial (Ayars et al, 1984; Sugahara et al, 1986; Donaldson et al, 1988b) and endothelial cell injury (Vandenbroucke-Grauls et al, 1987).

A role for leukocyte neutral proteinases in the pathogenesis of chronic inflammatory lung disease has been confirmed by the demonstration of emphysema in patients deficient in α1-PI, the major inhibitor of neutrophil elastase in the alveolar region (Laurell and Eriksson, 1963). Supporting evidence for the role of neutral proteinases in the pathogenesis of emphysema arose from the finding that cigarette smoke oxidant gases could cause oxidative inactivation of α1-PI and might thus contribute to the increased prevalence of emphysema in cigarette smokers (Janoff et al, 1980).

1.6.2. Consequences of extracellular matrix injury in inflamed lung

A primary function of the connective tissue matrix of the lung is to provide a scaffold for the cellular elements. However, in addition, the actual conformation of the connective tissue molecules in contact with the fixed lung cells may play a role in modulating the response of the lung to injury. The integrity of the basement membrane is essential for regeneration of normal tissue architecture *in vivo* (Vracko, 1972; Haschek and Witschi, 1979); this may be related to its function in acting as a support for replicating epithelial cells or to specific interactions between the cells and matrix which govern cellular proliferation (Lwebuga-Mukasa, 1986). Additionally, cell division and secretion of connective tissue molecules may be regulated by the specific configuration of the connective tissue (Martin and Kleinman, 1985). Several studies have shown altered properties of fibroblasts (Aplin and Foden, 1985), epithelial cells (Sugrue and Hay, 1981), smooth muscle cells (Holderbaum and Ehrhart, 1986) and endothelial cells (Ingber and Folkman, 1987) in response to connective tissue molecules and that work supports the theory of the regulatory role of lung connective tissue in maintaining normal tissue architecture. A further effect of connective tissue damage is the ability of connective tissue fragments to

enhance the inflammatory response as described in section 1.5.3. Thus, besides impairing the efficiency of gas exchange, damage to the connective tissue matrix of the alveolar septa in chronic inflammatory lung disease may also play a major role in the pathogenesis of the disease, by amplifying the inflammation and by interfering with the normal regulation of connective tissue turnover.

1.6.3. Epithelial/mesenchymal-cell interactions in inflamed lung

Studies of epithelial-mesenchymal cell interactions in embryology have indicated that direct cell-cell contact is an important factor influencing the normal growth and development of the foetal lung (Adamson and King, 1985). It has been suggested that the same mechanisms may be involved in the regeneration of epithelium on the alveolar surface following lung injury (Bhandari and Powell, 1987). The proliferative status of Type II epithelial cells is thought to be regulated (in part at least since macrophages may also be involved) by direct cell to cell contact between alveolar epithelial cells and the interstitial mesenchymal cells (Adamson and King, 1985). Chronic inflammatory lung disease is frequently associated with basement membrane damage which then permits increased contact between the alveolar epithelial cells and the mesenchymal cells of the septal interstitium. The extent of the epithelial-mesenchymal cell interactions is thought to be a predictor of the magnitude of subsequent mesenchymal cell proliferation (Witschi et al, 1980; Brody et al, 1981) and may also influence the production of connective tissue molecules by the interstitial fibroblasts. These mechanisms may be involved in the altered connective tissue and mesenchymal cell profile of chronically inflamed lungs. The alveolar lipoproteinosis and epithelial hyperplasia which characterises silicosis (Morgan and Seaton,1984) may also be associated with alterations in cell to cell and cell to matrix interactions following injury to the epithelium mediated by inflammatory leukocytes (Donaldson et al, 1987b).

1.6.4. The role of inflammatory leukocytes in regulating the inflammatory response in the lung

Leukocytes have several roles to play in modulating the inflammatory response in the alveolar region. These are bacterial killing, removal of effete cells (Newman et al, 1982; Duvall et al, 1985) and damaged connective tissue molecules (Carr, 1973; Etherington, 1980), and orchestration of the resolution of tissue injury. The latter is largely a function of inflammatory macrophages and is mediated by secretion of regulatory cytokines (Vaes,1985) and arachidonic acid metabolites (Henderson, 1987) as detailed in 1.5.4. Other regulatory molecules secreted by macrophages include a factor that increases monocytopoesis (FIMS) (Van Furth, 1988) and granulocyte/macrophage colony stimulating

factor (Piacibello et al, 1985), both of which enhance monocyte and neutrophil recruitment from the bone marrow. These molecules can act synergistically to stimulate or suppress macrophage, neutrophil and fibroblast function *in vitro*, depending on the culture conditions. They may thus play a central role in mediating connective tissue damage and deposition in lung disease. Inflammatory macrophages secrete various factors which can elicit fibroblast recruitment (Wahl and Wahl, 1981; Tsukamoto et al, 1981), proliferation (Leibovich and Ross, 1976; Bitterman et al, 1982), secretion of connective tissue molecules (Freundlich et al, 1986) and collagenase production (Huybrechts-Godin et al, 1985). Macrophages can also downregulate fibroblast function by secretion of regulatory molecules such as arachidonic acid metabolites (Metzger et al, 1986; Clark and Greenberg, 1987) and through the action of IL-1 and TNF (Elias et al, 1988). Epithelial cell proliferation is also modulated by macrophage cytokines (Kalina and Riklis, 1988). In addition, neutrophils secrete regulatory molecules such as IL-1 (Kusaka et al, in press) (see 1.5.5.) and produce factors which stimulate macrophage phagocytosis (Ishibashi and Yamashita, 1987) and production of arachidonic acid metabolites (Henderson, 1987). T lymphocytes may also be involved in the inflammatory response through secretion of gamma-interferon (1.5.6.).

All of the above may play a role in regulating the inflammatory response in the lungs. Thus, though the alveolar macrophage is considered to be the predominant regulatory cell in alveolar inflammation, other leukocytes may also contribute to the inflammatory response through secretion of cytokines.

1.7. Leukocyte proteinases in tissue injury during acute and chronic lung inflammation

Leukocyte proteinases play an important part in maintaining the normal structure and function of the lung parenchyma by their ability to kill and digest bacteria. They also digest and remove damaged host tissue prior to resolution or repair of injury and so mediate the normal turnover of lung connective tissue (Senior and Campbell, 1983). Under normal circumstances, the levels of proteinases in the alveolar region are low. This is because there are few leukocytes present and these are comprised largely of non-activated resident macrophages. During inflammation, the type and total number of leukocytes in the alveolar region changes as large numbers of inflammatory macrophages and neutrophils are recruited. This can lead to a gross increase in the burden of proteinase in the lung parenchyma. However, there are mechanisms in the lung which protect against proteolytic injury:- 1)Many of the leukocyte proteinases are released as zymogens or enzyme precursors which require activation before they can digest the tissue; 2)The antiproteinase screen of the lung (see 1.4.5.) forms a powerful defence against tissue

proteolysis by reacting with and inhibiting the majority of leukocyte neutral proteinases released into the lung parenchyma. Notwithstanding the effectiveness of these protective mechanisms, some "bystander injury" is an inevitable consequence of the accumulation of large amounts of leukocyte proteinases during an inflammatory episode in the lung. In acute inflammation, the tissue injury is resolved, with restoration of normal tissue architecture and function; this is despite the recruitment of large numbers of inflammatory leukocytes which may be up to 70% neutrophils in the case of some pneumonias (Begin et al, 1986a). In chronic inflammatory lung disease, by contrast, the magnitude of the inflammation is always less than in pneumonia, the leukocyte population in the bronchoalveolar lavage comprising as few as 5% neutrophils (Begin et al, 1986a; Rom et al, 1987). Thus the potential levels of proteinase and accordingly, tissue proteolysis, in the alveolar region during acute inflammation are much greater than in chronic inflammatory lung disease but pathological change generally accompanies only the latter. It appears, therefore that the duration, rather than the magnitude of the inflammation can be the most important factor in the development of pathological change. "Cumulative harm" may develop as each individual, small tissue insult in the chronic disease state, is followed rapidly and constantly by others, before the preceding injury has had time to resolve. This would ultimately lead to tissue derangements of much greater severity in chronic disease than in acute inflammation.

1.7.1. Initiation of proteinase secretion and potentiation of proteinase activity

Secretion of both acid and neutral proteinases is mediated largely by those processes which are involved in leukocyte activation (1.5.4; 1.5.5), some of which are listed in Table 2 below. In addition, secretion of neutrophil proteinases can be instigated by a peptide released from alveolar macrophages (Macarthur et al, 1987). Leukocyte proteinases, secreted as inactive enzyme precursors can be activated by neutrophil oxidants (Weiss et al, 1985), by an oxidant-independant process (Peppin and Weiss, 1986), by plasminogen activator (Werb et al, 1977) and other neutral proteinases (Horwitz et al, 1976).

Table 2. Factors inducing proteinase release by leukocytes

Phagocytosis (Werb and Reynolds 1974)

Chemotaxins (Weissmann et al, 1975)

Lipopolysaccharide (Khar, 1986; Fittschen et al, 1988)

Immunoglobulins and C3 (Takemura and Werb, 1984; Henson, 1974)

Concanavalin A (Khar, 1986)

Fibronectin breakdown products (Wachtfogel et al, 1988)

Colchicine (Werb, 1983)

Cyclic nucleotides (Weissmann et al, 1975)

IL-1 and TNF (Larrick and Kunkel, 1988)

Leukocyte proteinases, broadly classified as acid or neutral proteinases according to their pH optima, have many functions in common but may also have divergent roles to play in the milieu of inflamed lungs.

1.7.2. Leukocyte acid proteinases

The primary role of the acid proteinases is killing and digestion of microbes; this mainly occurs intracellularly, within the acid environment of the phagolysosome, but there is frequently some release of acid proteinases to the extracellular environment. This can occur following cell death or as a result of the phagocytic stimulus being too large to be fully internalised and thus the proteinases leak out of the incomplete phagolysosome (Fantone and Ward, 1984). Transport of acid proteinases to the extracellular environment, in response to specific stimuli, also occurs (Baggiolini and Dewald, 1985) and this may be a mechanism whereby microbial killing can take place in the extracellular environment if the concentration of the secreted lysosomal contents is sufficient to acidify the surrounding tissue (Davies and Bonney, 1980). Leukocyte-derived acid proteinases can damage connective tissue components of the lung (Vaes,1985) and so an unfortunate consequence of extracellular microbicidal events may be "bystander injury" to the surrounding host tissue. However, secretion of acid proteinases by inflammatory macrophages appears to be largely confined to the early stages of inflammation (Fantone and Ward, 1984). This may also be true of acid proteinase secretion by neutrophils and so the acid proteinases may not be a major source of tissue injury in chronically inflamed lung, although they may have a role to play in acute lung inflammation (Fels and Cohn, 1986). The neutral proteinases are thought to be more likely arbiters of extracellular matrix damage (Carp and Janoff, 1983) and recent work has concentrated on the role of these proteinases in such injury.

1.7.3. Neutral proteinases of neutrophils

The neutrophil neutral proteinases are largely contained within the azurophil granules of the cell (Baggiolini et al, 1980). With the exception of collagenase, which is a metalloproteinase contained within the specific granules in very small quantities, they are all serine proteinases and therefore susceptible to inhibition by the major inhibitor in the alveolar region, α1-PI (Travis and Salvesen, 1983). The neutrophil enzymes are generally considered to be preformed within the cells during maturation, prior to leaving the bone marrow (Havemann and Gramse, 1984) with no synthesis taking place thereafter,

except for plasminogen activator which is synthesised as required (Granelli-Piperno et al, 1977). However, there is one published report that intracellular elastase can be increased by up to 100% in human peripheral blood neutrophils triggered in vitro with lipopolysaccharide (Hart, 1984). The neutral proteinase content of neutrophils is considerably greater than that of inflammatory macrophages (Janoff et al, 1979; Gadek et al, 1984) and neutrophils have therefore been considered to be the major source of injurious proteolytic activity in inflamed lungs. The predominant neutrophil proteinase is elastase, a non-specific enzyme that can degrade most of the connective tissue molecules of the lung parenchyma including elastin, collagen, fibronectin and glycosaminoglycans (Senior and Campbell, 1983). The other major neutral proteinase of neutrophils is cathepsin G; this enzyme can also degrade a variety of connective tissue molecules and can act synergistically with elastase to potentiate tissue damage (Senior and Campbell, 1983). Elastase activity can also be enhanced by plasminogen activator, through its action in converting plasminogen to plasmin which is itself elastolytic(Chapman and Stone, 1984).

1.7.4. Neutral proteinases of macrophages

In comparison with neutrophils, inflammatory macrophages contain very low levels of neutral proteinases and no cathepsin G; the enzymes are not stored preformed in the cells but are synthesised and secreted in response to chemotactic, phagocytic or other inflammatory stimuli (Senior and Campbell, 1983; Gadek et al, 1984). The major macrophage neutral proteinases are plasminogen activator, collagenase and elastase and these enzymes appear to act in concert to degrade the extracellular matrix (Werb, 1983). With the exception of plasminogen activator which is regulated by specific inhibitors (Hart and Rehemtulla, 1988) the mechanisms governing regulation of macrophage-derived enzyme activity are not well characterised. Macrophage collagenase and elastase are metalloproteinases (Werb et al, 1980) and so are not inhibited by α1-PI but they are, however, readily inhibited by α2M. This factor may be of importance to the role of macrophages in tissue injury in chronically inflamed lung as discussed below.

1.7.5. Macrophage and neutrophil proteinases in tissue injury in inflamed lung

The relative contribution of macrophage and neutrophil proteinases to proteolytic injury of the alveolar septa has received little attention, since for the reasons described above, neutrophils are normally taken to be the main culprit in proteinase injury. Despite the greater content of neutral proteinases in neutrophils, compared with macrophages, proteinases from both cell types could have a role in eliciting tissue injury.

There are two ways in which neutrophil enzymes are likely to be active in injuring the alveolar septa, these are: (1) the concentration of proteinases may become so great, due to neutrophil influx and degranulation, that the antiproteinase screen becomes overloaded (Snider et al, 1986), or (2) at the site of close contact between the neutrophil and the septal cells or connective tissue components, an exclusion zone could be formed such that antiproteinases would not gain access (Johnson and Varani, 1981). Macrophage proteinases are secreted in lower amounts on a per cell basis than those of neutrophils but since inflammatory macrophages are generally present in much larger numbers than neutrophils in chronically inflamed lung (Janoff et al, 1979), the levels of macrophage proteinases may be increased sufficiently to render them of major importance to the disease process. In addition, because they are not inhibited by α1-PI, macrophage proteinases are more likely to persist in an active state in the alveolar region than those of neutrophils. Although there must be some means whereby released macrophage proteinases are inactivated in the lung there is no evidence to date, of a specific inhibitor of macrophage proteinases in the alveolar region. α2M readily inhibits macrophage proteinases and is secreted by macrophages, although in very low amounts. It is ubiquitously present in plasma but is a large molecule and does not cross the blood air barrier except as part of the inflammatory exudate during acute inflammation. Thus, in chronically inflamed lung, macrophage proteinases may contribute significantly to tissue injury.

1.7.6. Leukocyte neutral proteinases - proinflammatory and antiinflammatory effects in the lung

In addition to their role in microbial killing via secretion of toxic products, leukocyte neutral proteinases are involved in other aspects of host defense in the lung. These include roles in modulating the inflammatory response by leukocyte recruitment and activation and through tissue injury and repair during inflammation (see 1.5.2., 1.5.3. and 1.5.4.). Neutral proteinases can also function in other ways to enhance or suppress the inflammatory response. Secretion of plasminogen activator is thought to be a means whereby leukocytes can regulate further leukocyte recruitment through the proinflammatory effects of plasmin in the fibrinolysis, complement and clotting pathways (Hamilton, 1980). Neutral proteinases can enhance leukocyte recruitment by direct activation of complement components (Wright and Gallin, 1977; Johnston et al, 1976) and cleavage of C-reactive protein to its chemotactic products (Robey et al, 1987). Proteolysis of IgG produces a lymphocyte chemotaxin (Yamazaki et al, 1987) and α1-PI is a chemoattractant for neutrophils following proteolytic inactivation (Banda et al, 1988) which may be mediated by a neutrophil metalloproteinase (Desrochers and Weiss, 1988). Leukocyte neutral proteinases can also induce enhanced proteinase secretion (Werb and

Aggeler, 1978) and oxidant production (Johnston et al, 1985) and can initiate lymphocyte proliferation (Vischer et al, 1976). α2M-proteinase complexes have variously been shown to instigate (Vischer et al, 1980) or suppress neutral proteinase release (Johnston et al, 1982) and to stimulate α1-PI gene expression (Perlmutter et al, 1988). Prostaglandin synthesis by macrophages can also be regulated by neutral proteinases (Chang et al, 1980). From the foregoing studies it is apparent that leukocyte proteinases may play a major part in orchestration of the entire inflammatory response in the lung.

1.7.7. Leukocyte proteinases in human lung disease

The theoretical role of leukocyte proteinases in the disease process, gained from experiments in vitro, is given substance by the finding of increased levels of proteinase activity in bronchoalveolar lavage fluid and leukocytes from patients with chronic inflammatory lung disease. Elevated levels of collagenase have been demonstrated in bronchoalveolar lavage fluid of sarcoidosis patients (O'Connor et al, 1988) and patients with IPF (Gadek et al, 1979) and ARDS (Christner et al, 1985). Evidence of increased neutrophil elastase activity has also been demonstrated in bronchoalveolar lavage fluid of ARDS patients (Cochrane et al, 1983) and cigarette smokers (Janoff et al, 1983) and in the plasma of cigarette smokers (Weitz et al, 1987). In addition, in vitro studies of bronchoalveolar lavage leukocytes and peripheral blood neutrophils have also revealed increased proteinase activity in leukocytes from cigarette smokers (Hinman et al, 1980) and coalworkers (Hayem et al, 1980).

1.7.8. Rationale for the study of proteinases in experimental inflammatory lung disease

The foregoing section of the thesis has documented the variety of ways in which leukocyte proteinases can contribute to and sustain inflammation in chronically inflamed lungs and indicates that they are likely to play a major role in the disease process through these mechanisms. The role of inflammatory leukocytes in the disease process has been fairly well elaborated in other types of chronic inflammatory lung disease but little work has been done to investigate their role in the pneumoconioses. Leukocyte proteinases may be of particular importance to the development of the emphysematous lesions which frequently accompany the fibrotic changes in pneumoconiotic lungs. In addition, although much is known about the type and amount of specific proteinases secreted by leukocytes, there has been little work on the overall proteolytic activity of bronchoalveolar leukocytes. The rationale behind the work contained in this thesis was therefore to combine an investigation of the mechanisms of mineral dust-induced alveolitis with the development

of an assay which would measure the ability of viable bronchoalveolar leukocytes from dust-inflamed lungs to degrade connective tissue molecules.

1.8. Pathology of silicosis and coalworkers' pneumoconiosis

The main function of the lungs is gas exchange between the air and the blood. This involves the transfer of oxygen and CO_2 across the blood/air barrier and the structure of the alveolar tissue is fully evolved for this purpose (Burri, 1985). The two main features of lung tissue which contribute to the efficiency of gas exchange are:- 1) the honeycomb-like structure of the lung parenchyma which maximises the surface area of the lung and 2) the structure of the alveolar septa. The walls of the septa are very thin and so minimise the diffusion distance for oxygen and CO2 between the alveolar space and the pulmonary capillaries. In chronic lung disease, the efficiency of gas exchange becomes impaired when the structure of the lung tissue is altered by the disease process. The predominant types of lesion which occur in chronic lung disease are fibrosis and emphysema. These arise following the tissue damage that occurs in chronically inflamed lung. Such damage is currently considered to be mediated largely through the action of leukocyte-derived oxidants and proteinases.

Fibrotic lesions are formed by excessive or inappropriate deposition of connective tissue molecules and are characterised by thickening of the alveolar septa; in severe lesions this may occlude the entire alveolus. The increased connective tissue component also alters lung compliance and hence lung volumes are reduced. The major effect of such structural changes is the development of ventilation / perfusion mismatch and this is the major source of respiratory impairment in fibrotic lung disease. In addition, septal thickening increases the diffusion distance of the blood/air barrier and this may also contribute to inefficient gas exchange.

In emphysema, degradation of the connective tissue components of the alveolar wall leads to loss of alveolar septa. This results in a decrease in the number of attachments which, by traction, maintain the patency of small airways so these may close on expiration. The consequent airflow limitation is the main cause of dyspnea in emphysematous patients but additional contributing factors are the loss of surface area and elastic recoil of the lung tissue

The current hypothesis concerning the pathogenesis of fibrosis and emphysema suggests that despite their divergent outcomes these two pathological states originate in the same manner with the development of alveolar inflammation. The pathogenesis of the two disease processes is not yet fully elucidated but several additional hypotheses have been

proposed to explain the divergent pathological responses of the lung tissue. The balance between the destructive mechanisms which remove damaged or dead tissue and the regenerative mechanisms which replace that tissue is necessarily a fine one. Tilting the balance, even marginally in favour of repair mechanisms in chronic inflammatory lung disease, could lead, in the long term, to the excessive accumulation of connective tissue. Similarly, if tilted in favour of destructive processes, this would lead to the tissue loss which is manifest as emphysema.

1.8.1. Human studies of silicosis and coalworkers' pneumoconiosis

Silicosis and coalworkers' pneumoconiosis are chronic fibrotic lung diseases associated with exposure to silica and coalmine dust respectively (Morgan and Seaton, 1984). One of the earliest demonstrations of disease associated with the presence of siliceous material in the lungs at autopsy was published by Peacock in 1860; lung disease in coalworkers was first described around the beginning of the 1800s (reviewed in Meiklejohn, 1951). Since then, numerous other studies have also documented the relationship between silica and coalmine dust-exposure and disease (Morgan and Seaton,1984).

The lesions of silicosis and coalworkers' pneumoconiosis are similar and can be characterised on X-ray analysis by the International Labor Organization (ILO) classification (Guidelines-ILO,1980) which relates category of disease to number and size of lesions. At autopsy, the predominant lesions in both silicosis (Craighead et al, 1988) and coalworkers' pneumoconiosis (Davis et al, 1983) are fibrotic nodules. In coalworkers' pneumoconiosis, there is also evidence of extensive emphysematous change (Ruckley et al, 1984) and emphysema has been reported in silicotic lungs (Craighead et al, 1988). Impairment of lung function in these diseases is associated with the degree of pathological change; this is generally related to the extent of dust exposure but the disease may progress even after exposure has ceased (Ruckley et al, 1984). The prevalence of silicosis and coalworkers' pneumoconiosis in Great Britain has been markedly reduced in recent years by control of dust levels (Morgan and Seaton, 1984) but problems do still exist, particularly in the case of quarrying and the sandblasting of buildings, where there is currently no legislation governing permissible levels of dust exposure. In developing countries, occupational lung disease remains a major problem due to lack of effective dust control procedures. In China, for example, around 10 million people are thought to be exposed to silica and coalmine dust and between 600,000 and 800,000 pneumoconiosis patients were registered in 1987 (Sjostrand, 1989).

1.9. Factors contributing to the pathogenic potential of mineral dusts

1.9.1 Pathogenic effects of silica

The commonest form of silica used commercially is alpha quartz which has a hexagonal crystalline structure. Many recent studies have investigated aspects of the structure and reactivity of quartz particles to determine how they exert their harmful effects. The effects of crystal structure (Wiessner et al, 1988), particle number per unit weight (Ottery and Gormley, 1978), and free radical content (Vallyathan et al, 1988) have all been considered as possible factors modifying the biological reactivity of quartz. Current concepts of the pathogenicity of silica are related to the surface activity of the particles (Roggli et al, 1983). Crystalline silica is thought to form hydrogen bonds with the phospholipid, protein and/or lipoprotein constituents of the cell (Iler,1978) leading to cell death (Donaldson and Brown, 1988) or cell activation (Morgan et al, 1980). The release of inflammation-enhancing material into the alveolar lumen, subsequent to either of these cellular events would then cause recruitment of inflammatory leukocytes, with the attendant consequences for tissue injury as previously described (see 1.5-1.7).

1.9.2. Pathogenic effects of coalmine dust

In common with quartz, the pathogenic potential of coalmine dust has previously been attributed to its direct cellular toxicity (Gormley et al, 1980; Bowden and Adamson, 1984). However, the biological reactivity of coalmine dust, unlike quartz, is complicated by the fact that it is a mixed dust which contains many different minerals such as quartz, kaolin and mica, each of which may contribute to the pathogenic potential of the dust. The quartz content of coalmine dust has previously been considered to be of particular importance in this respect but *in vitro* studies of the cellular toxicity of coalmine dust have failed to establish correlations between quartz content and cytotoxicity (Gormley et al, 1980; Seemayer and Manojlovic, 1980). Epidemiology studies have indicated that there is no simple relationship between the quartz content of coalmine dust and its potential to cause pneumoconiosis (Walton et al, 1977; Davis et al, 1977). These studies are backed up by animal work which demonstrated a relationship between quartz content of dust and pneumoconiotic potential only at levels of quartz exceeding 10% (Ross et al, 1962; Rotaru and Lazarescu, 1982). Below 10%, the relationship between toxicity and quartz content is variable. Where the toxicity of the coalmine dust is less than would be predicted by the quartz content, the presence of minerals such as mica, though harmful in their own right (Le Bouffant et al, 1977), may confer some protection against the more actively toxic quartz. Such protection is thought to be mediated by the release of aluminium ions from

mica and/or other minerals, which then coat the quartz particles and so reduce their surface reactivity and biological activity (Le Bouffant et al, 1982). Evidence that the coal component of coalmine dust is harmful in itself comes from epidemiology data showing pneumoconiosis in anthracite areas, where the ash content of the coal is minimal (Hurley et al, 1982), and animal inhalation studies which show inflammation in response to anthracite (Donaldson et al, in press).

1.10. Previous experimental work investigating the role of inflammatory leukocytes in the development of pneumoconiosis

Leukocytes have long been considered to play a central role in the pathogenesis of the pneumoconioses. Initially, the resident alveolar macrophage was thought to play a key role in tissue damage through release of acid lysosomal proteinases following cell death or due to selective release of the enzymes. Much of the early work in this field concentrated on the *in vitro* effects of mineral dusts on alveolar (Morgan and Allison, 1980) or peritoneal macrophages (Davies et al, 1980), or on cell lines (Gormley et al, 1980).

With the advent of the technique of bronchoalveolar lavage it became apparent that, in chronic fibrosing lung diseases of varying aetiology such as sarcoidosis, ARDS and IPF, a common feature was the accumulation of large numbers of inflammatory leukocytes (both macrophages and neutrophils) in the bronchoalveolar region (Hunninghake et al, 1979). Functional studies of the bronchoalveolar leukocytes firmly implicated toxic products of these cells in the disease process (See 1.6.1.). Subsequently, the emphasis in research into the pneumoconioses shifted to assess the role of the inflammatory bronchoalveolar leukocytes in the disease process and revealed that alveolitis was a feature that was also common in these diseases (Voisin et al, 1985; Begin et al, 1986). Experimental studies similarly began to assess the alveolitis in dust-exposed animals; in the majority of cases, exposure by either intratracheal injection or by inhalation was used. The bronchoalveolar leukocytes were then retrieved by lavage, counted and various functional parameters studied. The earliest of this work was published by Bingham et al (1977) who found no increase in the numbers of leukocytes in the bronchoalveolar lavage in rats inhaling coalmine dust but substantial alveolitis following intratracheal injection of the dust. A number of groups reported similar increases in total cells in the bronchoalveolar lavage following intratracheal injection of silica in rats (Morgan et al, 1980), guinea pigs (Lugano et al, 1982), mice (Callis et al, 1985), rabbits (Stankus and Salvaggio, 1987) and sheep (Begin et al, 1987a). Similarly, alveolitis was also described following inhalation exposure of rats to silica (Bolton et al, 1986; Donaldson et al, 1988a) and coalmine dust (Donaldson et al, in press). An interesting feature of the inhalation studies was the finding

that the bronchoalveolar leukocytes were greater than 90% viable. This indicates that, as in the other chronic inflammatory lung diseases mentioned above, live cells are likely to be important in bringing about pathological change in pneumoconiosis. Leukocyte activation at the site of inflammation, with the attendant release of toxic products such as proteolytic enzymes is likely to play a key role in the disease process (Hunninghake et al, 1979). This theory is supported by the finding that bronchoalveolar leukocytes from patients with silicosis and coalworkers' pneumoconiosis secreted increased levels of elastase in vitro (Hayem et al, 1980). Increased levels of elastase and collagenase were also detected in the bronchoalveolar fluid in coalworkers' pneumoconiosis (Sablonniere et al, 1983). Experimentally, increased levels of elastase have been demonstrated in the lavage fluid of rats exposed to silica by intratracheal injection (Sykes et al, 1983). However, there has been no systematic study of the mechanisms of proteolytic damage in the lung following exposure to mineral dust. The purpose of the present study was therefore to investigate the role of leukocyte-derived proteinases in the pathogenesis of coalworkers' pneumoconiosis and silicosis.

1.11. Leukocyte proteinases and pneumoconiosis; the general hypothesis

The foregoing introduction has outlined the work and theories leading to the concept of a central role for leukocytes in fibrosis and emphysema. In deriving a general aim for the work described in this thesis an overview has been derived from many sources eg human work, work with experimental animals and also with cells in vitro. The findings that 1) some mineral dusts cause inflammation, 2) leukocytes accumulate in large numbers in inflamed lungs and 3) they have the ability to attack elements of the lung connective tissue led to the development of the general hypothesis to be tested in this work - namely that leukocytes accumulating in dust-exposed lungs have increased ability to break down connective tissue components.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. The experimental model

The purpose of this study was to investigate some of the mechanisms whereby particles deposited in the lung cause tissue injury, leading to permanent lung damage. To address these questions, rats were exposed to a selection of particles, administered by inhalation exposure or by injection directly into the lungs-intratracheal exposure. The leukocyte response to particle deposition in the lung was assessed by lavaging the resected lungs and counting the total number of leukocytes in the bronchoalveolar lavage fluid and by measuring the the proportions of macrophages, polymorphonuclear leukocytes and lymphocytes present. An assay of the ability of the bronchoalveolar leukocytes to proteolytically degrade extracellular matrix was developed and validated. The proteolytic activity of the bronchoalveolar leukocytes was assessed as a measure of their potential to inflict injury on the delicate tissue of the alveolar septa.

2.2. Animals

Rats were obtained from the breeding unit of the Institute of Occupational Medicine. The animals used were specific pathogen-free, syngeneic rats of the HAN or PVG strains, housed in RB3 cages (North Kent Plastics, Kent) and fed rat and mouse maintenance diet number 1; water was supplied ad libitum. The animal rooms were maintained at a constant temperature of 23-24°C, with a 12 hour photoperiod.

2.3. Particles

2.3.1. Particles used for intratracheal exposure

For the intratracheal experiments, the particles used were *Corynebacterium parvum* (*C.parvum*, Wellcome Reagents Ltd., Hither Green, London) which is a heat-killed bacterial preparation; zymosan (Sigma Ltd., Poole, Dorset)- a preparation of yeast cell walls; the mineral dust, titanium dioxide (TiO_2, Tioxide Ltd., Stockton on Tees) and the DQ12 quartz standard (quartz).

In some experiments the quartz particles were pre-treated with aluminium lactate according to the method of Begin et al (1987b) as follows:- 10mg quartz was mixed by rotation in 5ml of 1% aluminium lactate in distilled water for 3 hours at room temparature and was then washed twice in 10ml saline, with centrifugation at 3000rpm following each wash, to sediment the particles. (An MSE Super Minor centrifuge was used in this and all

other experiments in this study). The washed particles were then resuspended in saline to a concentration of 2mg/ml, prior to intratracheal injection of 0.5ml.

2.3.2. Coalmine dust used in the inhalation experiments

In the inhalation experiments, the dust used was a low-rank bituminous coalmine dust, collected from the air of a British colliery using dry fabric Bondina filters (Robertson et al, 1984). On ashing, the coal dust contained 18.2% kaolin, 0.0% mica and 6.7% quartz; these formed part of the 53.2% total ash in the dust. The dust was irradiated to kill microbial spores (150KRad) and was stored in a nitrogen atmosphere to minimise oxidation prior to use.

2.4. Exposure techniques

2.4.1. Intratracheal injection

Rats were anaesthetised by ether inhalation, the trachea was then exposed by dissection and a small incision made to allow access of a blunt cannula which was inserted as far as the first bifurcation of the bronchi. The particles were administered as a single bolus of 1mg in 0.5 ml saline or, in the case of *C. parvum*, 1.4 mg in 0.2 ml saline. Immediately following the injection, the skin over the incision was closed with two 11mm Michel clips (Albert Waeschle Ltd., Bournemouth) and the wound dusted with antibiotic powder (Cicatrin, Wellcome Foundation, London). The entire procedure was completed within 5 minutes and the animals recovered with no ill effects.

2.4.2. Inhalation exposure

Rats were housed in a $1m^3$ exposure chamber (Beckett, 1975) where they were exposed to the respirable dust at an airborne mass concentration of $10mg/m^3$. The coalmine dust used in this study was generated as a cloud using a dust generator (Wright, 1950) and was then passed into the chamber via the main ventilating airflow. The mass concentration of respirable dust in the chamber was checked daily by measuring the amount of dust collected onto the filters of a Casella MRE 113A dust sampler (Robertson et al, 1984). The average dust concentration in the chamber over the period of the study, was within ±3% of target (Donaldson et al, 1988c).

2.5. Lavage procedure for obtaining the bronchoalveolar leukocyte population from rat lungs

Rats were killed by phenobarbitone overdose, administered by the intraperitoneal route. The lungs and trachea were then exposed by dissection and the trachea cannulated with a

blunt 16g syringe needle. Following resection, the lungs were lavaged with four sequential 8ml volumes of saline at 37°C, with gentle massage of the lungs on each wash to increase the yield of cells. The recovered lavage fluid (5ml from the first lavage, 6-7ml thereafter) was pooled in plastic universal containers and placed immediately on ice. The bronchoalveolar lavage cells were pelleted by centrifugation at 1000rpm, washed once in 30 ml of ice cold phosphate buffered saline (PBS) and resuspended in ice cold tissue culture medium prior to use - the type of culture medium varied according to the assay procedure to be used thereafter.

2.6. Bronchoalveolar leukocyte total count and differential

Total number of leukocytes was estimated by counting in a Neubauer chamber. Cells were stained with trypan blue which confirmed that there were greater than 99% viable cells in all of the populations tested. The relative proportions of macrophages, polymorphonuclear leukocytes and lymphocytes were assessed by counting 100 cells per sample in May Grunwald-Geimsa stained cytocentrifuge smears.

2.7. Proteolysis assay

The method for assessing the proteolytic activity of the bronchoalveolar leukocytes was developed from the solid phase assay described by Campbell et al (1982) and detailed below. Leukocytes or their supernatants were incubated on a matrix of iodinated fibronectin, laminin or denatured collagen which was adhered in removawells (Dynatech Ltd., Billingshurst, Sussex) or microtitre plates (Flow Labs Ltd., Irvine).

2.7.1. Substrate iodination

Rat tail (type I) collagen, bovine type IV collagen and human plasma fibronectin (all from Sigma Ltd., Poole, Dorset) and mouse laminin (Gibco, Paisley) were iodinated by the method of McConahey and Dixon, 1966, modified by substituting L-cysteine for sodium metabisulphite. To 10µg substrate in 10µl 0.25M phosphate buffer, pH 7.5, was added 500µCi Na [^{125} iodine]. 10µl chloramine T (2.5 mg/ml in phosphate buffer) were added and mixed gently for 15 seconds. 100µl L-cysteine (0.6 mg/ml in phosphate buffer at a 1 in 5 dilution) were then added and again mixed gently before the final addition of 1ml potassium iodide (5 mg/ml in phosphate buffer). Free [^{125}iodine] was removed by chromatography on a G25 sephadex column (PD10, Pharmacia-LKB Ltd., Milton Keynes, Bucks) preequilibrated with PBS containing 0.05% bovine serum albumin (PBS/BSA)(BSA-Sigma Ltd, Poole, Dorset). The column was washed out with PBS/BSA and 0.5ml fractions collected. Fractions forming the first peak, which corresponded to the iodine-labelled substrate (Figure 1), were pooled and the radioactivity assessed by counting in a gamma

counter (Pharmacia-LKB); the labelled proteins showed activities of 5-25 $x10^6$ cpm/μg protein.

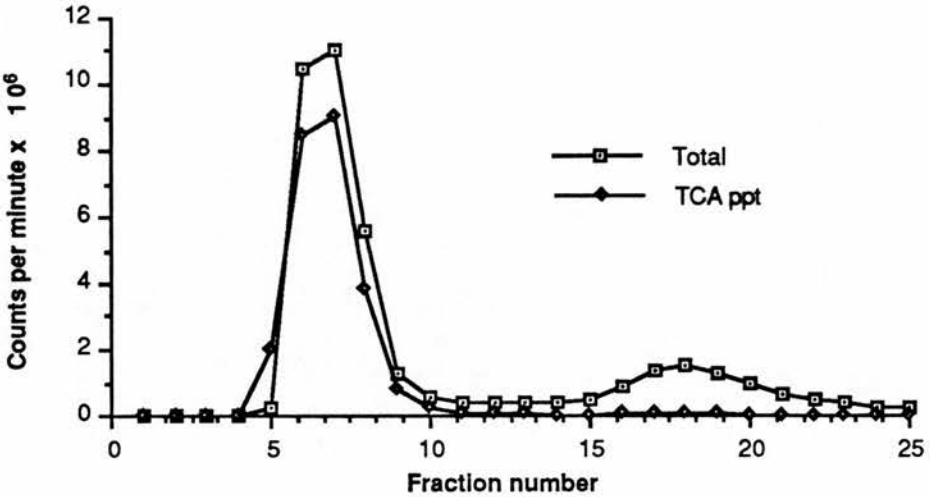

Figure 1. Verification of the efficiency of fibronectin radioiodination. Points represent total counts per minute in a 2μl aliquot of each 0.5ml fraction in a single iodination. The iodinated substrate is contained in the first peak with the second peak representing free [^{125}iodine]. The proportion of [^{125}iodine] that is protein-bound is represented by the plot of TCA precipitable counts in the same 2μl samples.

The iodinated collagens were diluted in 0.1M acetic acid and 100μl aliquots, containing 10,000 cpm for type I collagen and 60,000 cpm for type IV collagen were placed in microtitre removawells and dried at 45°C, a temperature which denatures the collagen molecules; iodinated laminin was diluted in saline to a concentration of 10,000cpm per 100μl which was then dried in removawells as above. Fibronectin was diluted to a concentration of 40,000 cpm/100μl saline for the validation experiments and 60,000 cpm/100μl thereafter and was dried onto wells of microtitre plates at 37°C. Drying of substrates onto plates took place for 3 days. Prior to use, the wells were presoaked for two hours with 200μl of the culture medium to be used in the assay and were then washed with 300μl saline to reduce background counts.

2.7.2. Trichloroacetic acid-precipitation of iodinated proteins

In the initial experiments, the efficiency of the substrate trace-labelling procedure was checked by trichloroacetic acid (TCA)-precipitation of each fraction obtained from the Sephadex column. A 5μl aliquot from each 0.5ml fraction was mixed in centrifuge tubes with 50μl serum and 2ml of 20% TCA was then added to precipitate the protein. The tubes were counted by gamma counter to estimate the total radioactivity present and were then spun at 2000rpm to pellet the precipitated protein. The supernatant was discarded and radioactivity in the pellet was compared with that in the whole sample prior to TCA

precipitation (figure 1). In the pooled fractions containing the radiolabelled protein, >80% of the pellet was TCA-precipitable, confirming that there was efficient trace-labelling of the protein.

2.7.3. Coating efficiency for adherence of [^{125}I]fibronectin onto microtitre plates

To determine the efficiency of attachment of [^{125}I]fibronectin to the wells, these were prepared in the normal way (see 3.7.1.). Using this method 60,000 counts per well of fibronectin were added to each well and dried on. The prepared wells were then presoaked in N&T medium for 2hrs at 37°C prior to washing with 300µl saline. The washed removawells were then counted by gamma counter to assess the total activity remaining and from this, the specific attachment was found, on average, to be 60% of the original counts added.

2.7.4. Proteolysis measurement

Cells, exogenous proteinases and inhibitors or triggers of proteolysis were added to the matrices in a final volume of 200µl of tissue culture medium. Triplicate wells were set up for each condition and were then incubated at 37°C; proteolysis was measured as amount of iodinated substrate released into a 150µl aliquot of the culture medium. Degradation of [^{125}I]-labelled substrate was measured using a gamma counter to determine counts per minute released. Apart from the experiment measuring the effect of increasing cell number/well on proteolysis of fibronectin, cells were added at 1×10^5 per well and in all but those experiments which investigated the time-response of the assay, the duration of the assay was 4 hours. In experiments to validate the proteolysis assay, the cells were cultured in Newman and Tytell's medium (N&T, this and all other media were obtained from Gibco-BRL Ltd, Paisley) containing 2% BSA (N&T/2%BSA). Thereafter, the medium used was Ham's F10 containing 2%BSA (F10/2%BSA), except in the assays of collagen and laminin proteolysis which were carried out in N&T alone.

2.8. Validation of the proteolysis assay

2.8.1. Proteolysis of fibronectin by exogenous enzymes

The ability of the assay to measure enzymic activity at neutral pH was assessed by measuring proteolysis of fibronectin, collagen and laminin matrices by exogenous bovine pancreatic trypsin (T8003), porcine pancreatic elastase (E0258) and bacterial collagenase (C5138) (all enzymes obtained from Sigma Ltd, Poole, Dorset; catalogue numbers given in brackets) at a concentration of 1mg/ml. To further standardise the assay, dose response

curves of the ability of the three enzymes to degrade fibronectin at concentrations ranging from 0.0001 to 1.0 mg/ml enzyme, were obtained using the fibronectin matrix.

2.8.2. The effect of specific proteinase inhibitors on fibronectin degradation by exogenous proteinases

The ability of the assay to distinguish serine and metalloproteinases was assessed in the fibronectin-proteolysis assay by determining the inhibitability of the proteinases by specific inhibitors. Inhibition of elastase (serine proteinase) and collagenase (metalloproteinase) by the serine proteinase inhibitor, α1-PI at 0.01, 0.1 and 1.0 mg/ml and the metalloproteinase inhibitor, disodium EDTA (EDTA - BDH, Glasgow) at 0.1, 1.0 and 10 mg/ml was measured. This was accomplished by adding the above concentrations of α1-PI and EDTA to elastase and collagenase (1mg/ml) in the fibronectin degradation assay.

2.8.3. Assessment of the effect of exogenous oxidants on the fibronectin matrix

To verify the finding that leukocyte-derived oxidants were not involved in fibronectin breakdown, exogenous oxidants were tested alone in the assay, at a concentration that would be secreted by the leukocytes. Hydrogen peroxide (30%, Sigma Ltd, Poole, Dorset) was diluted to a final concentration of 20 μMoles/well; 20μMoles of superoxide anion was generated in the wells by adding 40mMoles acetaldehyde and 20μg/ml xanthine oxidase.

2.9. The proteolytic activity of bronchoalveolar leukocytes

2.9.1. Dose-and time-response curves of fibronectin proteolysis by bronchoalveolar leukocytes

Dose and time-response curves were carried out to validate the proteolysis assay, using three bronchoalveolar leukocyte populations with markedly different cellular profiles - *C.parvum*-elicited, quartz-elicited, and control leukocytes. The cells were incubated on the fibronectin matrix at 0.001, 0.01, 0.1 and 1.0 $x10^5$ cells per well for 2, 4, 6 and 24 hours. The experiment was performed in triplicate.

2.9.2. Degradation of laminin and denatured collagen types I and IV by inflammatory leukocytes

The ability of control, *C.parvum*-elicited and quartz-elicited leukocytes to degrade laminin and denatured type I collagen, was compared in three separate experiments using microtitre plates coated with the iodinated substrates. The inflammatory leukocytes

(*C.parvum* and quartz) were tested at 0.01, 0.1 and $1.0x10^5$ cells/well; controls were tested at 0.01, 0.1, 0.5 and $1.0x10^5$ cells/well. Measurement of the proteolysis of type IV collagen was carried out in a single experiment using $1x10^5$ *C.parvum*-elicited leukocytes/well as lysed cells, cell supernatants and intact, viable cells. Lysed cells were prepared by freezing the cells rapidly in liquid nitrogen and thawing in a water bath at 37°C; this procedure was carried out three times for each cell suspension. Supernatants were prepared by culturing bronchoalveolar lavage leukocytes in microtitre wells for 4 hours; the supernatant medium was then harvested, centrifuged for 5 minutes at 2500 rpm to pellet cell debris and then tested immediately for ability to degrade denatured type IV collagen.

2.9.3. Proteolytic activity of bronchoalveolar leukocytes in the presence of inhibitors of oxidants and proteinases

The proteolytic activity of bronchoalveolar leukocytes was measured in the fibronectin proteolysis assay in the presence of the oxidant inhibitors, catalase and superoxide dismutase (SOD) (both from Sigma, Poole, Dorset) and the proteinase inhibitors, α1-PI and α2M. The oxidant inhibitor concentrations were 0.01, 0.1 and 1.0 mg/ml and the proteinase inhibitor concentrations were 0.01, 0.1 and 1.0 mg/ml for α1-PI and 0.005, 0.05 and 0.5 mg/ml for α2M. In one experiment the leukocytes were allowed to preadhere to the fibronectin matrix before the inhibitors were added to assess the effect of exclusion of inhibitors from the site of contact between cells and matrix.

2.9.4. The proteolytic activity of resident bronchoalveolar macrophages and of macrophages and neutrophils separated from a quartz-elicited bronchoalveolar leukocyte population

Separated populations of inflammatory macrophages and neutrophils were prepared from quartz-elicited bronchoalveolar leukocytes, by density gradient centrifugation, using Sepracell separating medium (Sepratech corporation, Oklahama, USA). Bronchoalveolar leukocytes from a quartz-exposed rat (approximately $20 x10^6$/rat) were washed, counted and resuspended in 9ml saline. Three ml of the leukocyte suspension was added to 6 ml Sepracell in a conical plastic centrifuge tube and mixed gently by inversion. The samples were then centrifuged at 2600rpm for 20 minutes at room temperature after which the cells appeared as two distinct bands; the macrophages were in a discrete layer at the top of the separating medium and the neutrophils were at the bottom of the tube. The cells were washed, counted and cytocentrifuge smears prepared for differential counting. The proteolytic activity of each population was then compared, using a [^{125}I]fibronectin matrix, with that of resident macrophages from the lungs of control rats.

2.10. Human leukocytes-preparation procedure

2.10.1. Human bronchoalveolar lavage leukocytes

Human bronchoalveolar leukocytes were obtained by saline lavage of a patient with a suspected chest infection who was undergoing routine bronchoscopy. The cells were washed once in saline prior to differential counting and testing in the proteolysis assay.

2.10.2. Human peripheral blood neutrophils

Venous blood from a healthy non-smoking volunteer was withdrawn into a syringe containing acid citrate dextrose. A leukocyte-rich plasma was obtained by Dextran (Travenol, Thetford, Norfolk); MW70,000) sedimentation, followed by centrifugation at 1200 RPM for 10 minutes, on a discontinuous plasma-percoll (Pharmacia, Uppsala, Sweden) (42 and 65%) gradient. This procedure resulted in a preparation whereby the mononuclear cells were found in a layer on the surface of the separating medium, the neutrophils were in a band that was 15mm below the mononuclear cells and the red blood cells pelleted at the bottom of the tube. The neutrophil band was aspirated and contaminating erythrocytes were lysed with 0.2% hypotonic saline, for 30 seconds, followed by reconstitution with 1.6% saline. The neutrophils were washed with PBS, total cells and differential were counted and the cells were then resuspended in medium for testing in the fibronectin proteolysis assay.

2.10.3. Human whole lung leukocytes

Whole lung leukocytes were obtained from a section of resected lung tissue which was distal to a tumour. The tissue was chopped finely with scissors and squashed gently with a glass homogeniser in a plastic universal. The finely minced tissue was then washed with PBS, several times through a 100 gauge nylon filter (John Stanair & Co. Manchester). The cells ($5X10^7$) were resuspended in 20 ml PBS and then separated by layering 3 ml cell suspension over 3.5 ml mono-poly resolving medium (Mono-poly - Flow Labs. Ltd., Irvine). They were then spun at 1400 rpm for 40 minutes and the layer which formed at the interface of plasma and separating medium was harvested. This contained a few contaminating red blood cells which were lysed with red cell lytic buffer (NH_4Cl-8.21g/L, $NaHCO_3$-0.84g/L, disodium EDTA-0.336g/L, pH 7.2). The cells were washed twice in PBS; total cells, differential count and proteolytic activity of the leukocytes were then assessed.

2.10.4. Assessement of the proteolytic activity of bronchoalveolar leukocytes and peripheral blood neutrophils

Proteolysis of fibronectin by the two leukocyte populations was measured at 0.01, 0.1 and $1.0x10^5$ cells/well. The ability of the two populations to respond to triggering was tested by including phorbol myristate acetate (PMA-Sigma Ltd, Poole, Dorset) in the assay at concentrations of 0.001, 0.01 and 0.1 μg/ml.

2.10.5. The proteolytic activity of leukocytes from excised lung tissue

The dose and time response of the proteolytic activity of leukocytes obtained by mincing excised lung tissue was measured in three separate experiments using the fibronectin proteolysis assay. Cells were tested at 0.001, 0.01, 0.1 and $1.0x10^5$/well for times of 2, 4, 6 and 24 hours.

2.11. Comparison of inflammatory responses in the bronchoalveolar region, elicited by different particles injected into the lung

The inflammatory response to 1.2mg of *C. parvum* or 1mg of zymosan, TiO_2 or quartz was measured following intratracheal exposure at intervals of 1, 3, 5 15 or 30 days after injection. Total cells in the lavage, differential count and the ability of the bronchoalveolar leukocytes to degrade fibronectin were measured at each time point. The ability of the bronchoalveolar leukocytes to respond to *in vitro* triggering was measured in the fibronectin proteolysis assay. The triggers used were PMA at 0.1, 1.0 and 10 μg/ml and zymosan, TiO_2 and quartz at 0.01,0.1 and 10 mg/ml. In some experiments, these were added into the proteolysis assay at the same time as the cells; in others, the leukocytes were cultured for three days in the presence of the triggers and proteolytic activity released into the supernatant medium was measured.

2.12. Coal inhalation expts.

Rats were exposed to $10mg/m^3$ of airborne coalmine dust (see 2.4.1.) for 7 hours per day, 5 days per week. On days 8, 16 or 32 of dust exposure, groups of 4 rats were removed from the chamber, killed and the bronchoalveolar leukocyte population obtained by lavage. At each time point, 2 control rats of identical age, maintained in room air were also lavaged. One group of four rats was removed from the chamber on day 32 of dust-exposure and maintained in room air for a further 64 days before being lavaged; these will be referred to as "recovery" animals. The inflammatory effect of the coalmine dust was measured as

total and percentage neutrophils in the bronchoalveolar lavage. Proteolytic activity of the bronchoalveolar leukocytes was measured in the fibronectin degradation assay. The proteolytic activity of leukocytes obtained 8 days after the start of dust exposure was measured in the presence of triggers and inhibitors of leukocyte proteolytic activity. The triggers used were PMA (0.1, 1.0 and 10 μg/ml) and zymosan (1,10 and 100 μg/ml); inhibitors were α1-PI (0.01, 0.1 and 1.0 mg/ml), α2M (0.005, 0.05 and 0.5 mg/ml), foetal calf serum (FCS-Gibco-BRL, Paisley;1, 5 and 10%) and EDTA (0.01, 0.1 and 1.0 mg/ml).

2.13. Assessment of the effect of aluminium lactate on the biological reactivity of quartz

Native quartz particles and quartz particles which had been pre- treated with aluminium (see 2.3.1.) were injected intratracheally. The inflammatory response to particle deposition and the proteolytic activity of the bronchoalveolar leukocytes were measured at intervals of one, four or twelve weeks thereafter. To address the mechanisms whereby the aluminium lactate might react with the quartz particles, two further experiments were performed: 1) 50μg of aluminium lactate in 0.5ml saline was administered to the rats by intratracheal injection, one month after injection of the quartz. Bronchoalveolar lavage of these animals was performed after a further 1 week or 1 month and the total and differential count and proteolytic activity of the leukocytes were measured, 2) To assess the specificity of the aluminium lactate treatment, rats were exposed to *C. parvum* and 50μg of aluminium lactate, administered at the same time. The inflammatory response was then measured as before, 1, 3 and 15 days after injection.

2.14. Histological preparations

Lungs treated with quartz or quartz/aluminium lactate were fixed after lavage by inflating with 10% formol saline prior to routine processing for histology. Whole lung sections were cut by microtome and stained with haematoxylin and eosin to visualise the tissue. Some of the sections were stained with reticulin to show up reticular collagen deposition.

2.15. Statistical analysis

Data from the early experiments validating the proteolysis assay with exogenous proteinases, and measuring dose and time responses for matrix proteolysis by bronchoalveolar leukocytes were evaluated by analysis of variance using the Minitab statistical package. In all other experiments, differences between means of treatment groups were tested by Student's "t" test.

RESULTS

3. RESULTS

3.1. Validation of the proteolysis assay

The aim of this thesis was to investigate the potential of inflammatory leukocytes to cause connective tissue damage in inflamed lungs. This question was addressed by measuring the ability of viable leukocytes to damage connective tissue molecules *in vitro*, through the action of neutral proteinases. The proteolytic activity of bronchoalveolar leukocytes was assessed by measuring their ability to degrade iodinated connective tissue components, adhered as a solid phase matrix in microtitre plates. The experiments in this part of the thesis were designed to develop and standardise the proteolysis assay prior to addressing the key questions of the thesis.

3.1.1. Matrix degradation by exogenous proteinases

Before commencing the leukocyte work, preliminary experiments were carried out to test the suitability of the proteolysis radioassay for detection of neutral proteinase activity. The proteolytic activity of exogenous collagenase, elastase and trypsin was assayed using iodinated laminin, fibronectin and denatured collagen matrices. The specificity of the assay in identifying different classes of enzyme was tested by measuring the ability of specific inhibitors of metalloproteinases and serine proteinases to inhibit proteolysis of fibronectin by collagenase (metalloproteinase) and elastase (serine proteinase).

3.1.1.1. Proteolysis of fibronectin, laminin and denatured collagen by exogenous proteinases

Microtitre plates coated with iodinated laminin, fibronectin or denatured type I collagen were incubated with 1mg/ml of collagenase, elastase or trypsin in F10/2%BSA. Each enzyme caused significant proteolysis of all matrices ($p<0.005$ compared with medium alone) (Table 3). Elastase was the most active and collagenase the least so but this does not reflect the true relative proteolytic activity of the three enzymes, since they were assayed according to weight and not enzyme activity.

Table 3. Proteolysis of laminin, fibronectin and denatured collagen, matrices by exogenous collagenase, elastase and trypsin (1mg/ml).

	SUBSTRATE		
ENZYME	Denatured Collagen	Fibronectin	Laminin
Collagenase	2963(165)	4736(1311)	2904(726)
Elastase	3193(394)	7170(529)	3761(367)
Trypsin	2947(181)	6092(743)	3622(217)
None	885(156)	1399(119)	655(102)

Results are presented as counts of [125I]-degradation products released and are the mean(sd) of three wells per sample in two separate experiments.

The proteolytic activity of all three enzymes was dose-dependant as shown in Table 4, using a fibronectin matrix and, in a further single experiment, was abolished by dilution to low concentrations (0.1,0.01,0.005 and 0.001 mg/ml, Figure 2).

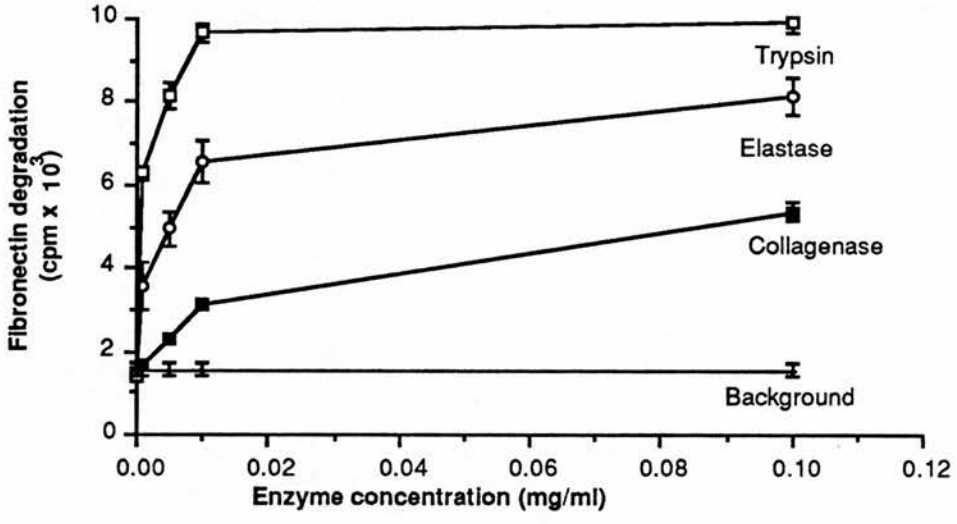

Figure 2. Dose response of fibronectin proteolysis by exogenous enzymes; enzyme concentrations are given in mg/ml. Results are the mean(sd) of triplicate wells in four separate experiments.

Table 4. Dose-dependent proteolysis of fibronectin by exogenous collagenase, elastase and trypsin. Results are the mean(sd) of three wells per enzyme concentration in three separate experiments.

	ENZYMES		
Dose(mg/ml)	Collagenase	Elastase	Trypsin
0.01	3859(669)*	7245(647)	9805(332)
0.1	6063(635)	8667(945)	10844(905)
1.0	7076(524)	10600(890)	11601(872)

* *Background counts (no enzyme, 1539(160)) have not been subtracted from the absolute counts.*

The increase in proteolysis with increasing enzyme concentration was significant at all concentrations for collagenase and elastase ($p<0.005$). Trypsin had greater proteolytic activity than collagenase or elastase at 0.01 and 0.1 mg/ml but showed less of a dose-dependant change, the only significant increase in proteolysis being between 0.01 and 0.1 mg/ml ($p<0.01$). The lack of a significant dose response with trypsin, which had the greatest activity at all concentrations, may be because at that enzyme activity, the plates were near to the limit of their capacity to detect proteolysis.

3.1.1.2. Inhibition profile of collagenase and elastase

Inhibition of 1mg/ml collagenase and 1mg/ml elastase by EDTA at 0.1, 1.0 and 10 mg/ml and by α1-PI at 0.01, 0.1 and 1.0 mg/ml was measured using the fibronectin matrix (Figure 3).

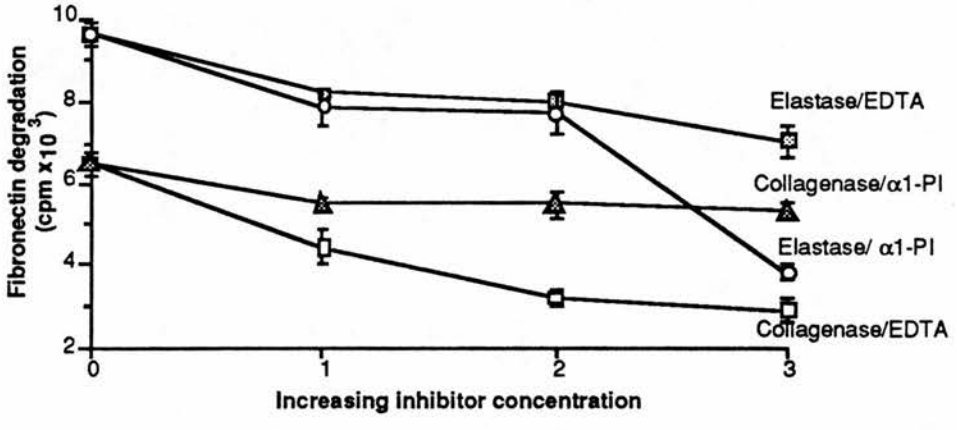

Figure 3. Dose response of the metalloproteinase inhibitor, EDTA and the serine proteinase inhibitor, α1PI in reducing fibronectin proteolysis by the exogenous metalloproteinase, collagenase and the serine proteinase, elastase. Results are the mean(sd) of triplicate wells per sample in two separate experiments. Inhibitor concentrations:- α1-PI, 0.01, 0.1 and 1.0 mg/ml; EDTA, 0.1, 1.0 and 10 mg/ml

EDTA caused dose-dependant inhibition of collagenase that was significant at all concentrations (0.1mg/ml $p<0.01$, 1.0 mg/ml $p<0.005$, 10 mg/ml $p<0.0005$) but the reduction in elastase activity which occurred only at 10 mg/ml EDTA was not significant. Conversely, there was no significant reduction of collagenase activity at any concentration of α1-PI but elastase activity was significantly inhibited by 1.0 mg/ml α1-PI ($p<0.0005$).

3.1.2. Matrix degradation by bronchoalveolar leukocytes

The assay was further standardised by using in the main, three bronchoalveolar leukocyte populations. These populations were control cells, *C.parvum*-elicited cells and quartz-elicited cells (Figure 4a-c). These were of varying neutrophil content (Table 5) and since neutrophils constitutively contain greater levels of neutral proteinases than macrophages, were therefore likely to contain different amounts of neutral proteinase. TiO_2-elicited bronchoalveolar leukocytes obtained 5 days after intratracheal injection, were also used in some of the experiments in this part of the study; this TiO_2 population contained 2% neutrophils.

The dose and time response of the proteolytic activity of control and inflammatory leukocytes was established using a [^{125}I]fibronectin matrix. Proteolysis of iodinated laminin and denatured type I, and type IV collagen matrices by the leukocytes was also assessed using a single concentration of the cells.

Table 5. *Differential count of bronchoalveolar leukocytes from control,* C.parvum*-elicited and quartz-elicited rats. Results are the mean(sd) of 100 cells counted for each group in six separate experiments.*

	DIFFERENTIAL COUNT %		
	Macrophages	PMN	Lymphocytes
Control	99(1)	0	1(1)
Quartz	49(11)	48(12)	3(2)
C.parvum	22(7)	78(7)	0

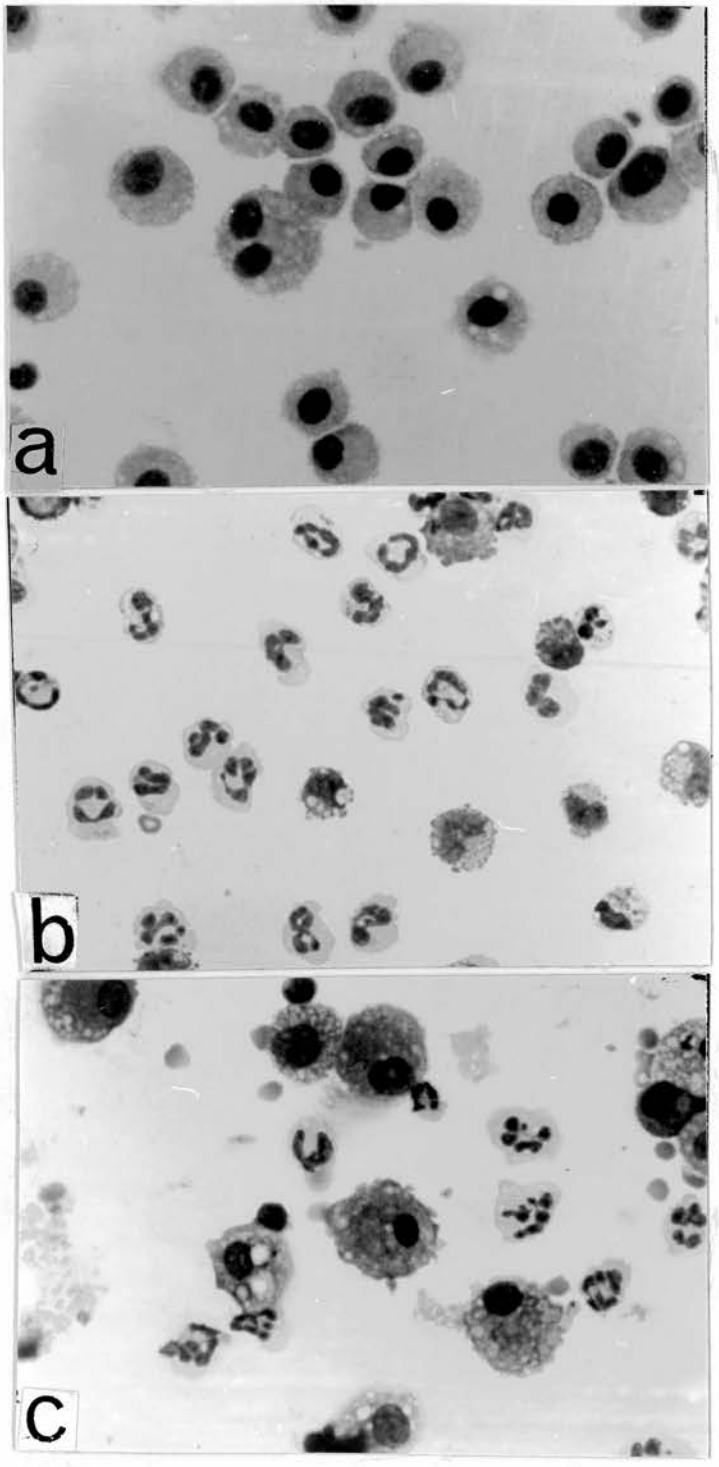

Figure 4. Bronchoalveolar leukocytes from a.) control rats, b.) rats exposed to C.parvum *for 16 hours prior to lavage or c.) rats exposed to quartz 5 days prior to lavage.*

3.1.2.1. Proteolysis of fibronectin by bronchoalveolar leukocytes

The ability of all three populations to degrade a fibronectin matrix increased with increasing cell number (Figure 5); this was true at 2, 4, 6 and 24 hours (Table 6) but only the 6 hour time point is presented in the figure for clarity.

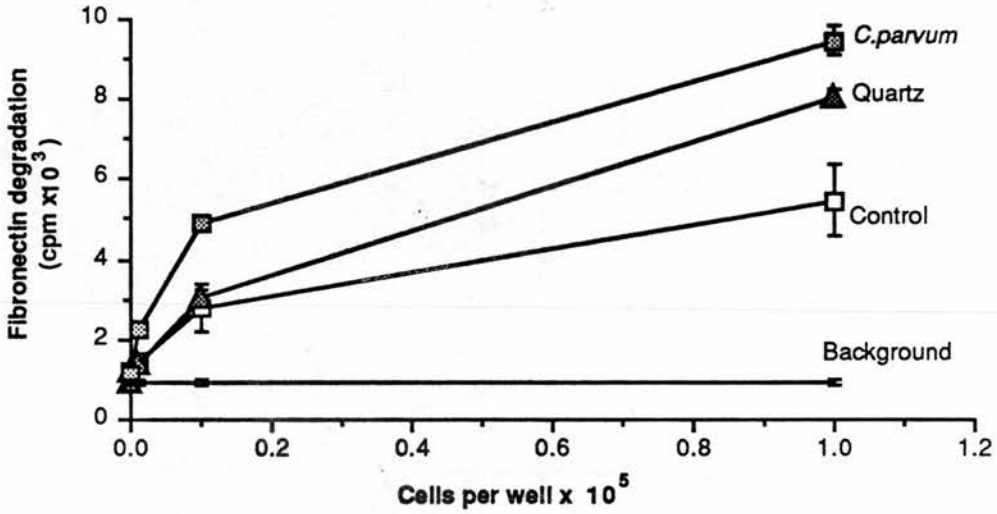

Figure 5. Dose response of fibronectin proteolysis by bronchoalveolar leukocytes (6 hour time point). Results are the mean(sem) of triplicate wells per sample in three separate experiments.

At 6 hours, the release of [^{125}I]fibronectin-degradation products by control and quartz populations was significantly greater than background at concentrations of 0.1 and 1.0 x 10^5 cells/well (controls $p<0.0025$ and $p<0.005$ respectively; quartz $p<0.005$). The difference between the control and quartz leukocytes reached statistical significance at 1.0 x 10^5 cells/well ($p<0.0005$). *C. parvum*-elicited cells were significantly more active than control leukocytes at 0.01 x 10^5 cells/well and above ($p<0.005$). The *C. parvum*-elicited cells consistently had greater proteolytic activity than the quartz cells but the difference between the two populations reached statistical significance only at 0.1 x 10^5 cells/well ($p<0.005$).

Proteolysis of fibronectin by control, *C. parvum* and quartz-elicited cells increased with time (Figure 6). Only the results obtained using 1x10^5 cells/well are shown in the figure for clarity but the time response was also present at 0.01 and 0.1 x10^5 cells per well (Table 6).

Table 6. Dose and time response of fibronectin proteolysis by control, C.parvum *and quartz-elicited leukocytes. Results are the mean(sd) of three wells per sample in three separate experiments.*

Leukocyte population	Time (hrs)		Number of cells/well ($x10^5$) 0	0.001	0.01	0.1	1.0
Control	2	$\bar{x}$	532	613	603	781	1781
		sd	145	171	120	112	412
Control	4	$\bar{x}$	745	831	892	1112	2548
		sd	84	148	157	274	669
Control	6	$\bar{x}$	916	1223	1473	2769	5453
		sd	149	176	511	1754	2646
Control	24	$\bar{x}$	1647	1962	1975	2827	5901
		sd	240	283	418	1006	2035
C.parvum	2	$\bar{x}$	532	657	927	2305	5458
		sd	145	123	101	294	983
C.parvum	4	$\bar{x}$	745	841	1585	3603	7605
		sd	84	88	103	450	990
C.parvum	6	$\bar{x}$	916	1142	2232	4915	9419
		sd	149	248	242	264	1102
C.parvum	24	$\bar{x}$	647	2695	4643	8785	13599
		sd	240	230	328	876	923
Quartz	2	$\bar{x}$	532	612	759	1184	3396
		sd	145	85	136	153	832
Quartz	4	$\bar{x}$	745	1020	1163	2393	5876
		sd	84	92	96	487	798
Quartz	6	$\bar{x}$	916	1183	1403	3069	8031
		sd	149	129	150	576	492
Quartz	24	$\bar{x}$	647	2639	3849	8607	11728
		sd	240	212	367	745	853

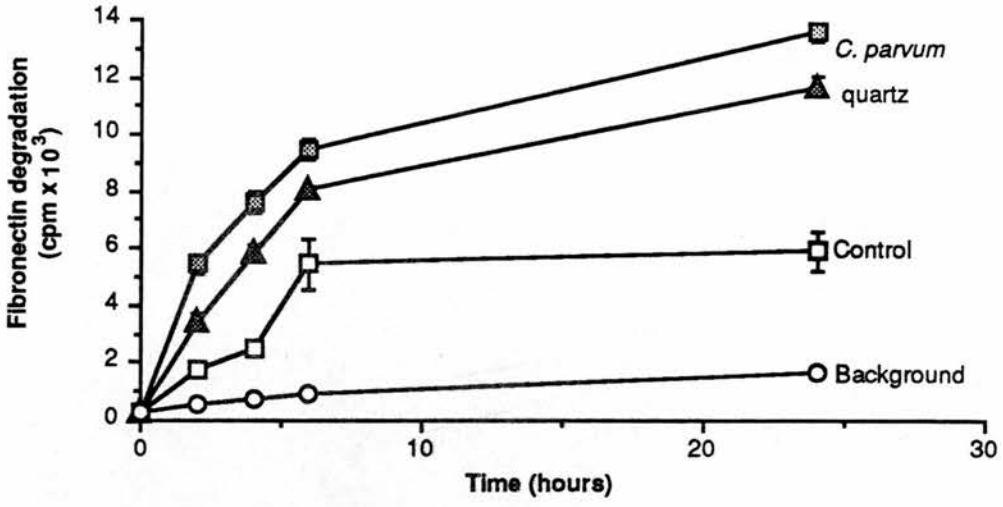

Figure 6. Time response of fibronectin proteolysis by bronchoalveolar leukocytes ($1x10^5$/well). Results are the mean(sem) of triplicate wells per sample in three separate experiments.

At 1 x 10^5 cells/well each population caused significantly ($p<0.025$-$p<0.005$) greater release of counts than medium alone at every time point. Both inflammatory populations were significantly more active than controls at each time point ($p<0.01$-$p<0.005$) and similarly, the *C.parvum* cells were significantly more active than the quartz cells at all time points ($p<0.025$-$p<0.005$).

3.1.2.2. Proteolysis of denatured collagen and laminin

All three cell populations had dose-dependent activity in degrading denatured collagen and laminin matrices (Figure 7A and 7B).

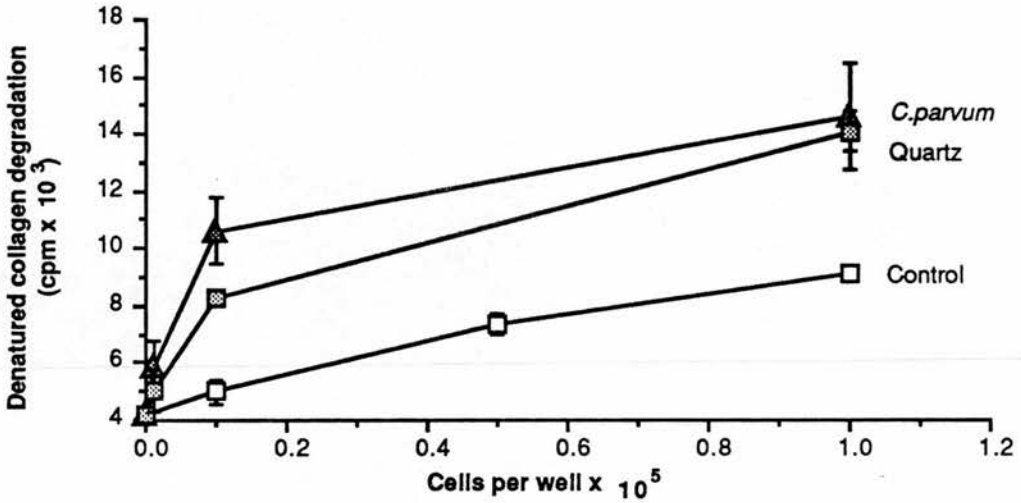

Fig 7a

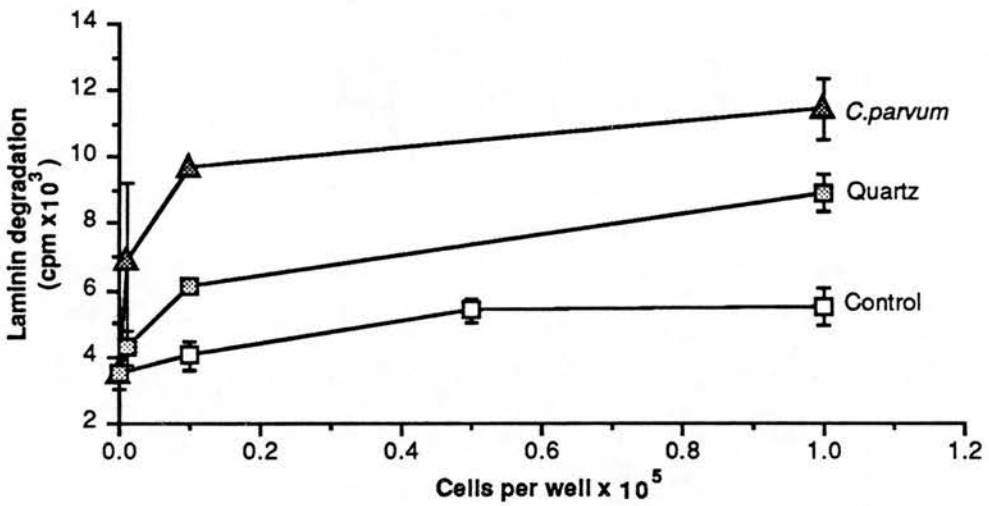

Fig 7b

Figure 7a and b. *Dose response of denatured collagen (fig a) and laminin (fig b) proteolysis by bronchoalveolar leukocytes at 1x10^5/well. Results are the mean(sd) of triplicate wells in a single experiment.*

In [^{125}I]collagen-coated microtitre plates, at 1x10^5 cells/well, counts released into the supernatant medium by all three populations, were significantly greater than those released by medium alone (controls $p<0.01$, *C.parvum* and quartz $p<0.001$). Using [^{125}I]laminin-coated plates the results were similar at 1x10^5 cells/well (significantly different from medium alone:- controls, *C.parvum* and quartz $p<0.001$). Proteolysis of both matrices by the *C.parvum* and quartz leukocytes was also significantly greater than

background at $0.1x10^5$ cells/well ($p<0.001$) but there was no significant difference between medium alone and control leukocytes at $0.1x10^5$ cells/well. Quartz and *C.parvum* leukocytes had significantly greater ability to degrade both matrices than control leukocytes at 0.01, 0.1 and $1.0x10^5$ cells/well ($p<0.005$). The *C.parvum*-elicited leukocytes were more active in degrading both types of matrix than the quartz cells at all concentrations but the difference did not reach statistical significance. Thus, the three populations had different proteolytic activity which reflected, in part, the number of neutrophils in each.

3.1.2.3. Proteolysis of type IV collagen by intact inflammatory leukocytes, lysed cells and cell supernatants

C.parvum-elicited inflammatory leukocytes caused marked proteolysis of type IV collagen (5336[76])(mean[sd]) (Figure 8) which was significantly greater than background (1838(43), $p<0.001$).

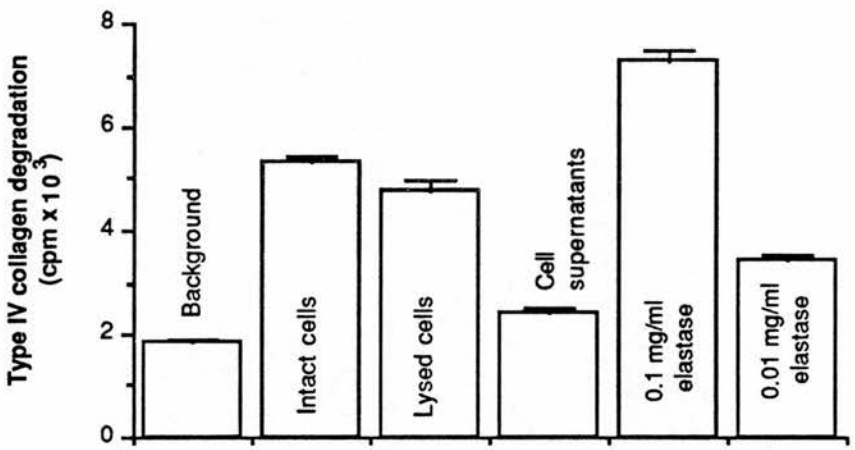

Figure 8. Proteolysis of type IV collagen by C.parvum*-elicited bronchoalveolar leukocytes as intact cells, lysed cells or 24 hour supernatants of the cells. Results are the mean(sd) of triplicate wells in a single experiment and are compared with the proteolytic activity of porcine pancreatic elastase tested in the same experiment.*

The activity of the *C.parvum* cells was equivalent to between 0.1 and 0.01 mg/ml elastase (7306(208) and 3426(115) respectively). Intact leukocytes had significantly greater proteolytic activity than lysed cells (4768(158), $p<0.01$). Leukocyte supernatants were produced separately in a 4 hour period, under the same conditions as those used in the assay. These had significantly less ability to degrade fibronectin (2412[136]) in 4 hours, than intact leukocytes or lysed cells ($p<0.0005$).

3.1.3. Summary

The experiments presented in this section of the thesis served to demonstrate that the experimental protocol was sound. The techniques of intratracheal injection and bronchoalveolar lavage yield leukocytes of different cellular and functional profiles depending on the injected material and are therefore valuable tools in studying the leukocyte response of the lung to deposited exogenous material. The proteolysis assay is capable of detecting the main enzymes of interest, that are likely to be of importance in causing proteolytic damage in the lung ie collagenase and elastase. The two enzymes can be distinguished by their different inhibition profiles in response to specific inhibitors of serine and metalloproteinases. The assay also distinguishes the activity of different bronchoalveolar leukocyte populations in a cell number- and time-dependent manner. Inflammatory bronchoalveolar leukocytes were more active than controls in degrading fibronectin, collagens type I and IV and laminin. The proteolytic activity of intact cells was greater than that of lysed cells (which contained all cell fragments) or cell supernatants which suggests that part of the activity of intact leukocytes may be due to de novo enzyme synthesis over the 4 hours of the assay.

3.2. The effect of inhibitors of oxidants and proteinases on fibronectin degradation by bronchoalveolar leukocytes

Both oxidants and proteinases have hitherto been shown to damage connective tissue molecules and so the role of these leukocyte-derived products in causing proteolysis of fibronectin by control, quartz and *C.parvum*-elicited leukocytes was assessed by the use of specific inhibitors.

3.2.1. The effect of antioxidants on fibronectin degradation by control and inflammatory bronchoalveolar leukocytes

To assess the role of leukocyte-derived oxidants in fibronectin proteolysis, leukocytes were cultured on a fibronectin matrix in the presence of the antioxidants superoxide dismutase and catalase; these scavenge superoxide anion and hydrogen peroxide respectively. There was no significant reduction in fibronectin proteolysis by either superoxide dismutase or catalase at concentrations of 0.01, 0.1 or 1.0 mg/ml (Figure 9).

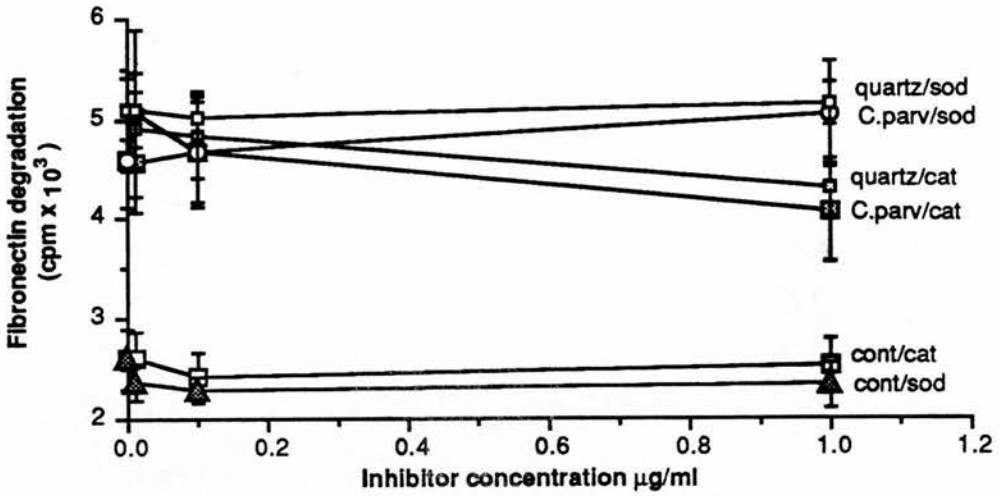

Figure 9. Proteolysis of fibronectin by control, C.parvum *and quartz-elicited bronchoalveolar leukocytes in the presence of the hydrogen peroxide inhibitor, catalase and the scavenger of superoxide, superoxide dismutase. Results are the mean(sem) of triplicate wells per sample in three separate experiments. (cat = catalase, sod = superoxide dismutase)*

This result suggested that leukocyte-derived oxidants played no part in matrix degradation in this assay. Exogenous hydrogen peroxide and superoxide were therefore tested in the assay. A dose of 20µM was used for each oxidant as this is approximately the amount that would be produced by leukocytes in culture. The exogenous oxidants also failed to cause matrix degradation (medium alone-2986[85])(mean[sem]counts per minute [^{125}I]fibronectin degraded); superoxide dismutase-2058(56); hydrogen peroxide-1858(78)) and thus confirmed the finding that leukocyte-derived oxidants are not involved in matrix degradation in this assay.

3.2.2. The effect of proteinase inhibitors on fibronectin proteolysis by control and inflammatory bronchoalveolar leukocytes

To assess the role of individual proteinases in matrix degradation, the inhibitor profile of the leukocytes was obtained using α1-PI and α2M, two inhibitors which are present in varying amounts in the alveolar region of the lung. In most of the experiments assessing the inhibitor profile of the bronchoalveolar leukocytes, the cells and proteinase inhibitors were added together to the wells. However, leukocytes in the lung are likely to be in close contact with the connective tissue matrix. It is possible that at the site of close contact between cells and matrix, an exclusion zone might be formed where molecules of large molecular weight, including proteinase inhibitors, could not gain access. In order to test this hypothesis, leukocytes from control, TiO_2- or quartz-treated rats were used. The bronchoalveolar leukocytes were preincubated on a fibronectin matrix prior to addition of α1-PI and then inhibition of matrix proteolysis was measured as previously.

3.2.2.1. Inhibitor and leukocytes added to wells at the same time

The proteinase inhibitors, α1-PI and α2M minimally inhibited control cell proteolysis of fibronectin; the inhibition was neither significant nor dose-dependant. Both inhibitors did cause inhibition of the two inflammatory populations (Table 8) when added at the start of the assay. α1-PI was equally effective in inhibiting both the *C.parvum* and the quartz cells, with significant reductions of proteolysis at 0.1 and 1 mg/ml ($p<0.001$) and at 0.01 mg/ml ($p<0.01$). α2M also significantly reduced fibronectin proteolysis by the quartz-elicited cells at 0.05 mg/ml ($p<0.05$) and at 0.5 mg/ml ($p<0.001$) but was less effective in reducing *C.parvum*-mediated proteolysis of fibronectin, achieving a significant reduction only at a concentration of 0.5 mg/ml ($p<0.001$).

Table 8. Degradation of fibronectin by control, C. parvum *and quartz bronchoalveolar leukocytes in the presence of the proteinase inhibitors, α1-PI and α2M. Results are the mean(sem) of triplicate samples in three separate experiments.*

		LEUKOCYTE POPULATIONS		
Inhibitor	Conc.(ug/ml)	Control	*C.parvum*	Quartz
0		2603(299)	4587(456)	5108(304)
α1-PI	0.01	2380(251)	2885(569)**	3963(374)**
	0.1	2428(261)	2795(379)***	3260(335)***
	1.0	2333(235)	2097(230)***	2531(274)***
α2M	0.005	2787(321)	4298(418)	4648(335)
	0.05	2456(281)	4157(571)	4198(348)**
	0.5	2286(288)	3528(418)***	3534(275)***

** *Significant reduction compared with no inhibitor, $p<0.01$*
*** *Significant reduction compared with no inhibitor, $p<0.001$*

Background counts (no cells, 1679(69)) have not been substracted. N.B. The inhibitor profiles of *C.parvum* and quartz leukocytes were assessed at different times so, because of half life effects with the [^{125}I], the proteolytic activity of each is not directly comparable.

3.2.2.2. Preadherence of leukocytes to a fibronectin matrix: effect on the inhibitory capacity of α1-PI

When control, TiO_2- and quartz-elicited leukocytes were added to a fibronectin matrix at the same time as α1-PI this led to a significant inhibition of proteolysis over the 4 hours of the assay with each of the populations ($p<0.001$) (Figure 10).

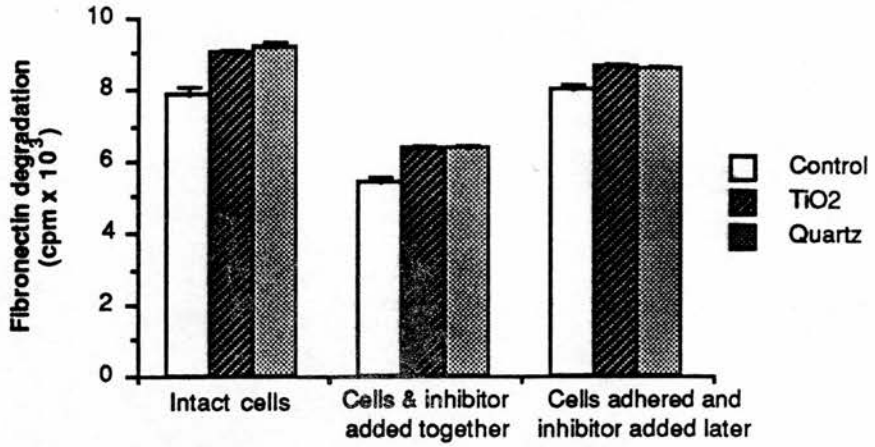

Figure 10. Effect of preadhering TiO_2 and quartz-elicited bronchoalveolar leukocytes to the fibronectin matrix, on the inhibitory capacity of α1-PI in the proteolysis assay. Results are the mean(sem) of triplicate wells in a single experiment.

However, when the leukocytes were allowed to preadhere to the matrix for 30 minutes prior to addition of α1-PI, there was no significant reduction in the proteolytic activity of any of the cells in the subsequent 4 hours.

3.2.3. Summary

The foregoing results indicate that leukocyte-derived oxidants have no role to play in matrix degradation in the proteolysis assay. Instead the results implicate leukocyte proteinases as the major arbiters of matrix degradation. The inhibitor profiles of the *C.parvum*-elicited leukocytes (>80% neutrophils) and the quartz-elicited leukocytes (50% neutrophils, 50% macrophages) suggest that their proteolytic activities are similar and likely to be mediated by serine proteinases. In the quartz population, around 50% of the population is alveolar macrophages which do not secrete serine proteinases. Some of the serine proteinase activity may be accounted for by newly recruited monocytes which secrete serine proteinases and some may be due to membrane associated cathepsin C but further work is required to clarify this. The finding that leukocytes preincubated on the matrix were less susceptible to inhibition of their proteinases by α1-PI suggests that leukocytes could retain their proteolytic activity, even in the presence of a sufficiency of antiproteinase in the alveolar region, due to exclusion of high molecular weight inhibitors from the site of cell:matrix attachment.

3.3. The proteolytic activity of human leukocyte populations

The proteolytic activity of peripheral blood neutrophils and alveolar leukocytes, obtained by bronchoalveolar lavage or by extraction from excised lung tissue, was compared using a fibronectin matrix.

3.3.1. Dose- and time-response curves of proteolysis by leukocytes from excised lung tissue

Leukocytes obtained from excised human lung (>95% macrophages) showed dose- and time-dependent proteolysis of fibronectin (Figure 11).

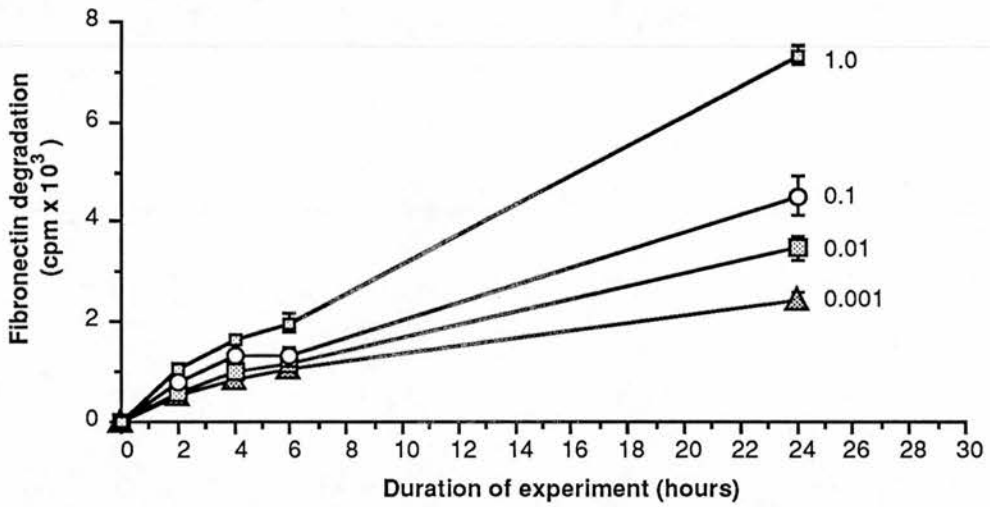

Figure 11. Cell number response of fibronectin proteolysis by leukocytes obtained from resected human lung tissue. Results are the mean(sem) of [^{125}I]fibronectin breakdown products released in triplicate wells in a single experiment with increasing number of cells per well (x10^5).

Release of counts was significantly greater than background at all time points at $1.0x10^5$ cells/well ($p<0.01$) but did not reach statistical significance at any other dose, except for $0.1x10^5$ cells/well at 4 hours ($p<0.005$).

3.3.2. Comparison of the proteolytic activity of bronchoalveolar lavage leukocytes with peripheral blood neutrophils and the response of each population to exogenous triggers in vitro

Peripheral blood neutrophils were obtained from a healthy volunteer. They were >95% neutrophils and showed dose-dependent proteolysis of fibronectin after 4 hours which was significantly greater than background at 0.1 and $1.0x10^5$ cells/well ($p<0.01$ and $p<0.0005$ respectively) (Figure 12).

The bronchoalveolar leukocytes were obtained from an ex-smoker who had not smoked for 1 year at the time of lavage but who was suspected of having a current chest infection. The differential count of the bronchoalveolar leukocytes was 86% macrophages, 1% neutrophils and 13% lymphocytes and the cells were greater than 98% viable as assessed by trypan blue exclusion. The bronchoalveolar leukocytes had no significant proteolytic activity even at the maximum concentration of $1.0x10^5$ cells/well.

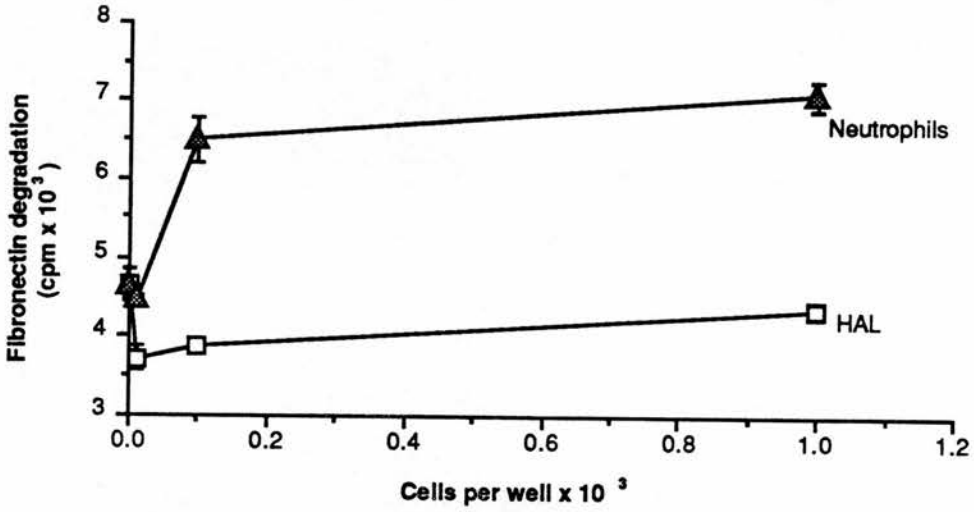

Figure 12. Dose response of fibronectin proteolysis by human bronchoalveolar lavage leukocytes(HAL) and peripheral blood neutrophils from a different individual. Results are the mean(sem) of triplicate wells in a single experiment.

When the assay was performed in the presence of the exogenous trigger, PMA, only the peripheral blood neutrophils demonstrated increased proteolytic activity (Figure 13).

There was a significant and dose-dependent increase in the proteolytic activity of the neutrophils ($p<0.0005$ compared with cells alone) but no effect on the bronchoalveolar leukocytes.

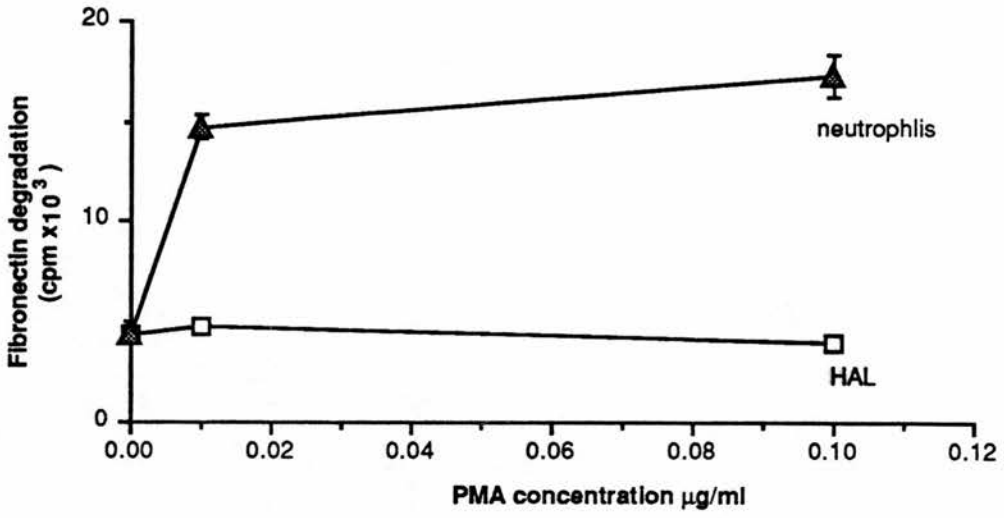

Figure 13. Response of human bronchoalveolar lavage leukocytes (HAL) and peripheral blood neutrophils to triggering with PMA. Results are the mean(sem) of triplicate wells in a single experiment.

3.3.3. Summary

Leukocytes obtained from excised lung tissue, at a site distal to a tumour showed dose- and time-dependent proteolytic activity but those obtained by lavage from the lungs of an ex-cigarette smoker with a current infection had no such activity; *in vitro* triggering of the bronchoalveolar lavage leukocytes with PMA failed to stimulate any increase in their proteolytic activity. The failure of *in vitro* stimulation to elicit increased proteolytic activity of bronchoalveolar leukocytes has been demonstrated several times, with different cell types in the course of the work for this thesis (3.5.4; 3.6.5). Bronchoalveolar leukocytes may be refractory to stimulation because of the unique position they occupy in the body where they are exposed to a continuous insult of exogenous material. Untriggered peripheral blood neutrophils had intrinsic proteolytic activity but they also responded to an exogenous stimulatory signal by increased proteolysis of fibronectin. Activation of peripheral blood neutrophils by the separation procedure cannot be ruled out as a possible explanation of their intrinsic proteolytic activity. However, their ability to respond to further triggering *in vitro* indicates that they were not maximally triggered and suggests that these cells may exhibit different levels of activation depending on the stimulus. The responsiveness of peripheral blood neutrophils to *in vitro* triggers may reflect a requirement for efficient responses to challenge, at various sites in the body, by these cells. Differences between the alveolar leukocyte populations *in vitro* may be related to the methods of retrieval of the cells. The increased proteolytic activity of the minced lung cells may be due to the presence of interstitial leukocytes, which are more active than lavage cells, in the minced lung preparation. Alternatively, the differences between the two lung

leukocyte preparations may reflect true differences in the activational status of leukocytes from the bronchoalveolar region in different disease states *in vivo*.

3.4. Comparative proteolytic activities of resident and inflammatory bronchoalveolar macrophages and exudate neutrophils

All of the foregoing results have demonstrated that the numbers of both macrophages and neutrophils are increased in inflamed lungs. Neutrophils contain considerably more neutral proteinases than macrophages and although they do not synthesise proteinases once they have left the bone marrow, they are thought to be a major source of proteinase in inflamed lungs. Macrophages, however, are capable of enzyme synthesis and may play a part in increasing the proteinase burden of the lung during chronic inflammation when they are frequently the predominant cell type. Additionally, macrophage enzymes, being metalloproteinases are not susceptible to inhibition by α1-PI and so are more likely to remain active in the extracellular fluid of the lung. The relative contribution of each cell type to connective tissue proteolysis has therefore been investigated by obtaining bronchoalveolar leukocytes from inflamed lungs and separating them by differential centrifugation. The proteolytic activity of macrophages from inflamed and control lungs has been compared with the activity of inflammatory bronchoalveolar neutrophils.

3.4.1. Separation of inflammatory macrophages and neutrophils

Inflammatory leukocytes were obtained by lavage from rat lungs 30 days after intratracheal injection of 1mg of quartz. Total numbers of leukocytes in these animals was ninefold greater than in the controls and comprised around 50% macrophages and 50% neutrophils (Table 9). The cells were separated into inflammatory macrophage and neutrophil populations by differential centrifugation and the total cells and differential count in the two populations was assessed.

Table 9. Total cells and differential count in lavage of control or quartz-treated rats and in the separated quartz populations.

	Total cells ($\bar{x}$(sd)x10^6)	DIFFERENTIAL COUNT (%) Mac.	Neut.	Lymph.*
Control	1.8(0.3)	97	0	3(1)
Quartz	16(2.2)	49(6)	50(13)	1(1)
Quartz macrophages	6.1(2.8)	93(2)	4(2)	3(2)
Quartz neutrophils	8.3(2.7)	16(6)	85(7)	3(1)

* *Mac. = macrophages; Neut. = neutrophils; Lymph. = Lymphocytes*

Results are presented as the mean(sem)x10^6 of total cells and mean(sem)% of each cell type in lavage. There were three rats per group in four separate experiments for the control and whole quartz populations; the cells were pooled in each experiment before preparing the separated cells, so the data for the quartz macrophages and neutrophils is the mean(sem) of a single separation in four experiments. The percentage recovery following the separation procedure was 80-90%.

3.4.2. Proteolytic activity of inflammatory macrophages and neutrophils

The proteolytic activity of the separated, quartz-elicited bronchoalveolar leukocytes and of control macrophages was assessed in four separate experiments, using the fibronectin proteolysis assay. Each of the cell populations released significantly more degraded fibronectin into the supernatant medium than background which was 1628(161) counts per minute (mean(sem)) ($p<0.005$) (Figure 14).

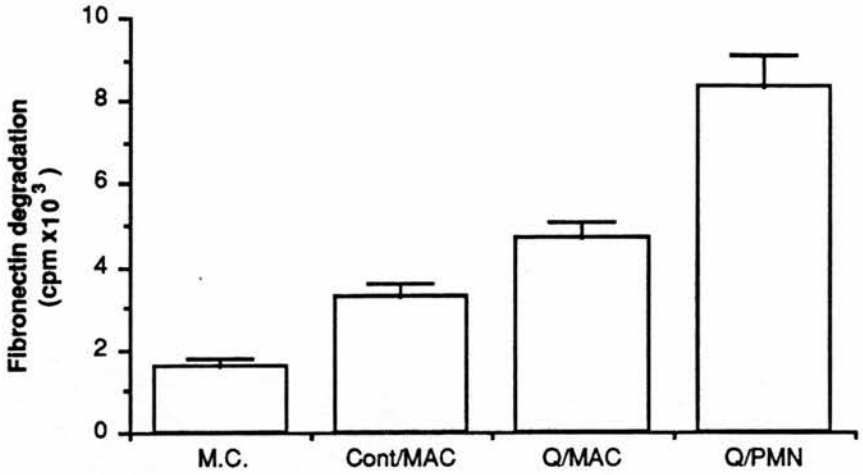

Figure 14. Comparative proteolytic activities of bronchoalveolar leukocytes:- Cont/MAC=control, Q/MAC=macrophages separated from quartz-inflamed lung, Q/PMN=neutrophils separated from quartz-inflamed lung, MC=medium control. Results are the presented as counts per minute of degraded fibronectin and are the mean(sem) of triplicate wells in five separate experiments.

Inflammatory macrophages (4700[387]) and neutrophils (8328[805]) were both significantly more active than control macrophages- (3250[338]) ($p<0.01$ and $p<0.0005$ respectively). Although the inflammatory macrophages were more active than controls, they were significantly less active than the inflammatory neutrophils ($p<0.005$). The increased proteolytic activity of the inflammatory macrophages, compared with controls cannot be explained by the 4% contamination of neutrophils since that would be expected to increase the proteolytic activity of the macrophages by only 12%. In effect, the actual increase in proteolysis over control macrophages was 45%.

3.4.3. Macrophage activation in the peritoneal cavity

In order to substantiate the finding that inflammatory bronchoalveolar macrophages had enhanced proteolytic activity, intraperitoneal injection of tryptose phosphate broth was used to produce different populations of inflammatory leukocytes in rats and in mice. Such treatment elicited no increase in total cells and no change in the differential count in either species (Table 10) but the proteolytic activity of the inflammatory macrophages was increased compared with the resident macrophage population.

Table 10. Total cells and differential count of leukocytes retrieved from the peritoneal cavity 3 days after intraperitoneal injection of tryptose phosphate broth.

		DIFFERENTIAL COUNT (%)			
	Total cells ($x10^6$)	Mac.	Neut.	Lymph.	Eos.*
Control mouse PECS	9.4	83	1	13	3
TP broth mouse PECS	10.8	86	1	11	2
Control rat PECS	6.6	79	1	3	17
TP broth rat PECS	8.0	87	1	0	12

* *Mac. = macrophages; Neut. = neutrophils; Lymph. = Lymphocytes; Eos = Eosinophils; PECS = peritoneal exudate cells; TP broth = tryptose phosphate broth.*

Results are based on a single rat or mouse and are expressed as total cells $x10^6$ and percentage of each cell type in the peritoneal population.

The proteolytic activity of the tryptose phosphate broth-elicited peritoneal macrophages was modestly but significantly greater than the resident macrophages for in both rat and mouse cells ($p<0.001$ and $p<0.005$ respectively, Figure 15).

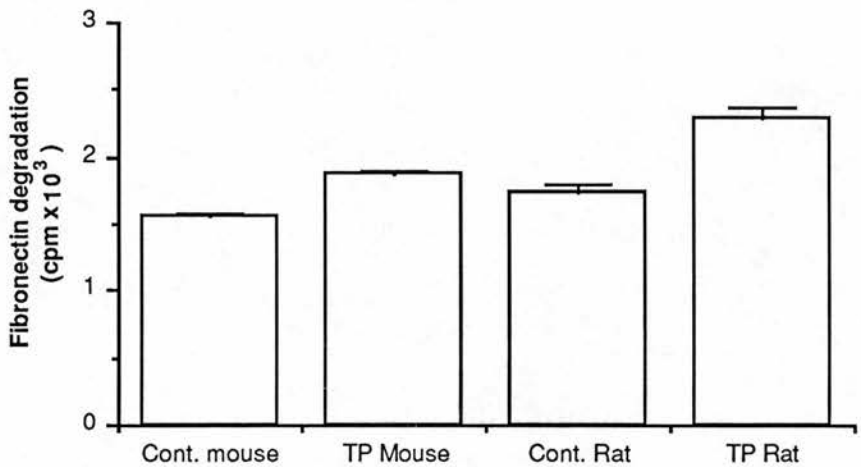

Figure 15. Fibronectin proteolysis by inflammatory macrophages obtained from the peritoneal cavities of rats and mice injected with tryptose phosphate broth. Cont. mouse = control mouse macrophages; TP mouse = tryptose phosphate broth-elicited mouse macrophages; Cont. rat = control rat macrophages; TP rat = tryptose phosphate broth-elicited rat macrophages. Results are presented as counts per minute of degraded fibronectin and are the mean(sem) of triplicate wells in a single experiment.

This supports the earlier finding (3.4.2) that inflammatory macrophages can exert enhanced proteolytic activity.

3.4.4. Summary

The increased proteolytic activity, on a per cell basis, of inflammatory bronchoalveolar leukocytes from quartz-dosed rats is due largely to the presence of inflammatory neutrophils which are at least twice as active as resident or inflammatory macrophages in causing fibronectin degradation. However, macrophages can also be activated to secrete increased proteinase in response to an *in vivo* signal. The increased activity of the inflammatory macrophages, together with the markedly increased cell numbers in lavage may make a substantial contribution to the proteinase burden in the inflamed lung parenchyma.

3.5. The magnitude and duration of the inflammatory response to particles deposited in the lung by intratracheal instillation

The initial inflammogenic potential of particles depositing in the lung is only one determinant of their potential to inflict damage on the lung tissue and other factors are likely also to contribute to that harm. In particular, the biodegradeability of particles is probably of major importance in this respect. Therefore, in this section of the work, the initial inflammogenicity and durability of particles has been related to the duration of the tissue response which they evoke in the lung. The potential role of these two parameters in lung damage was assessed by measuring the magnitude and duration of the lungs' response to particles of different durability and pathogenic potential, administered by intratracheal instillation. This exposure method is preferable in this type of study, since it allows a direct comparison of particles and can be manipulated to give information on the effects of particle size, number, surface area or mass. In this study the comparisons were based on the mass of particles administered. A comparison has been made between the alveolitis elicited by two different biodegradable (and hence non-durable) particles and that evoked by the non-toxic, durable mineral dust, TiO_2 and the harmful, durable mineral dust, DQ_{12} quartz.

3.5.1. Total number of leukocytes retrieved by bronchoalveolar lavage

All of the particles provoked a transient increase in the number of leukocytes in the bronchoalveolar lavage one day after injection (Figure 16), which was most with *C. parvum* (26.7[1](mean[sd])x10^6) and least with TiO_2 (11.4[1.7]); the total number of leukocytes in the bronchoalveolar lavage in each treatment group was significantly greater than in the pooled controls (4.3[0.7]) at this time point ($p<0.001$). (The pooled control data was obtained by taking the mean of the control data for all time points).

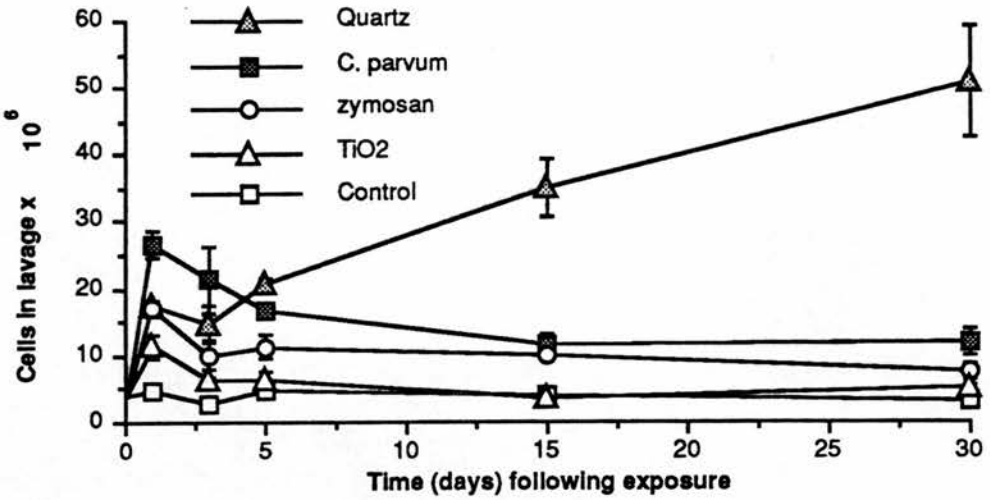

Figure 16. Total cells in bronchoalveolar lavage with time following intratracheal injection of the indicated particles. Results are the mean(sem) of three rats per group at each time point.

With the exception of quartz, the alveolitis declined rapidly, approaching background levels by 3 days in the TiO_2-treated animals (6.3[0.6]) and 30 days in the zymosan-treated animals (7.7[1.2]). Leukocyte numbers also approached background levels by 30 days in those animals treated with *C.parvum* (11.9[2.1])(mean[sd] % neutrophils in lavage). The response to quartz, however, though similar in magnitude to the zymosan response at 1 day (17.3[0.9] and 17.0[1.0] respectively), showed only a slight decrease at 3 days; thereafter, the number of leukocytes in the quartz bronchoalveolar lavage escalated and, by 30 days was 10-fold greater (51.0[8.3]) than in the pooled controls (4.3[0.7]).

3.5.2. Percentage of neutrophils in bronchoalveolar lavage of control and treated rats

The percentage of neutrophils in the bronchoalveolar lavage reflected a typical acute inflammatory response (Figure 17) for most of the particles, with a peak of neutrophil recruitment 1 day after the intratracheal injection; there were significantly more neutrophils in lavage of the treated animals than in the controls at this time point ($p<0.005$).

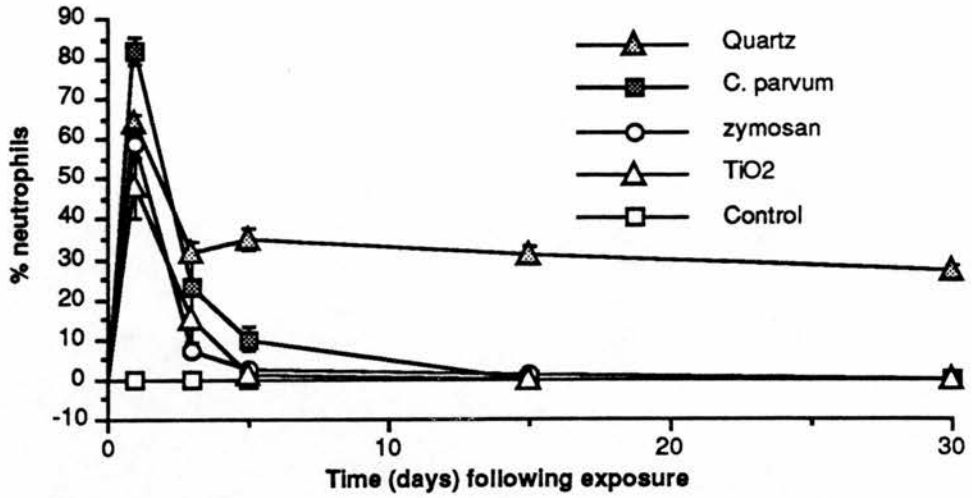

Figure 17. Percentage of neutrophils in lavage fluid with time following intratracheal injection of the indicated particles. Results are the mean(sem) of three rats per group at each time point.

The least influx of neutrophils was seen in the TiO_2-treated rats (48[7])(mean[sd]%) and the greatest, in those dosed with *C.parvum* (82[3]). The percentage of neutrophils in the bronchoalveolar lavage leukocytes subsided rapidly, returning to around background levels (0) within 5 days for TiO_2 (1[0.1]) and zymosan (2.6[0.3]) and 15 days for *C.parvum* (0). In the quartz-dosed animals, however, although the percentage of neutrophils decreased from a peak of 64(2.4) at 1 day to 32(2.1) at 3 days, it then persisted at around 30% for the remaining 27 days of the experiment.

3.5.3. Fibronectin-degrading activity of bronchoalveolar leukocytes

The proteolytic activity of the bronchoalveolar leukocytes reflected the cellular response (Figure 18), being significantly greater than the controls at 1 day with all of the treatments ($p<0.001$) and, with the exception of quartz, returning rapidly to normal thereafter.

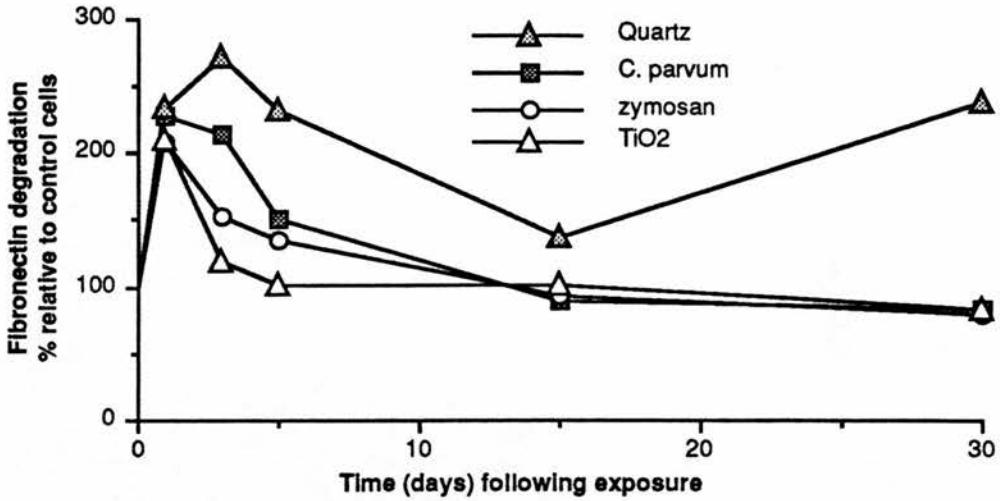

Figure 18. Proteolytic activity of the indicated inflammatory leukocytes with time following intratracheal injection of particles. Results are expressed as counts per minute of degraded fibronectin released into the culture medium/1x10^5 cells and are the mean(sem) of three wells per sample and three animals per treatment group at each time point.

The quartz-elicited leukocytes had 6-fold greater activity than the controls at 1 day and this was still 5-fold greater than the controls at 30 days, when all the other populations had returned to control levels.

The proteolysis assay measures the proteolytic activity per cell but an additional factor influencing the overall protease burden in the lung is the total number of inflammatory leukocytes present. Accordingly, the proteolysis data per cell was multiplied by the total number of leukocytes in the bronchoalveolar lavage (Figure 19) to gain an indication of the potential total protease burden in the bronchoalveolar region in arbitrary units.

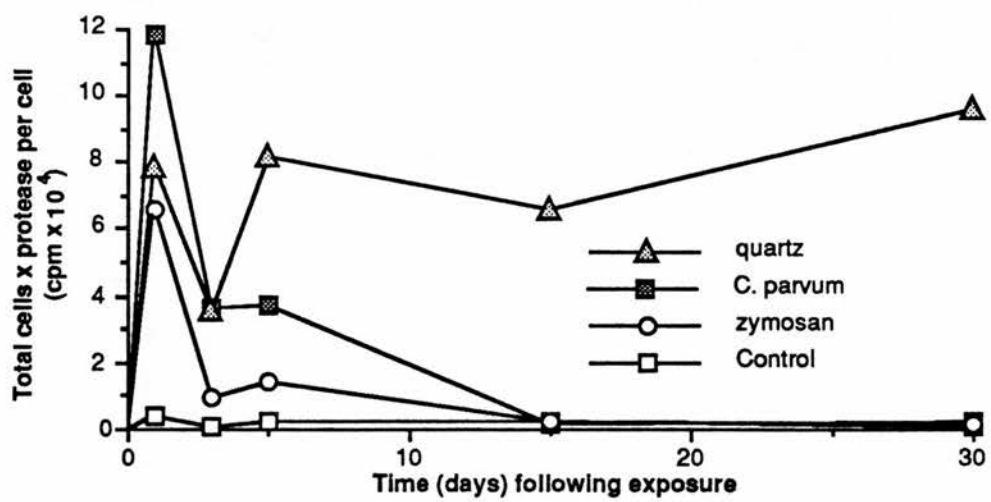

Figure 19. Proteolytic activity per cell multiplied by total number of cells in the bronchoalveolar lavage to give an indication of the potential overall proteolytic burden in arbitrary units in the alveolar region. Results are the mean of three rats per treatment group at each time point.

This data showed that 1 day after treatment, the C.parvum rats had the greatest overall potential for proteolytic injury in the lung; the total proteinase burden in these animals was 31 times that of the controls. However, this was rapidly superceded by the proteinase levels in the quartz-treated animals where, by 30 days after treatment, the total proteinase burden in the alveolar region was 72 times that of the controls. So, the potential for proteolytic injury in the lungs of the quartz-treated rats was considerably greater than in any other treatment group with respect to the increased proteolytic activity per leukocyte, the total number of leukocytes and the duration of the alveolitis.

3.5.4. *In vitro* stimulation of the proteolytic activity of bronchoalveolar leukocytes

None of the bronchoalveolar leukocyte populations, harvested 5 days after exposure *in vivo*, showed any increased proteolysis of fibronectin when the assay was performed in the presence of TiO_2, quartz or zymosan particles or PMA (Table 11).

Table 11. *Effect of* in vitro *triggering, over a four hour period, on fibronectin proteolysis by bronchoalveolar leukocytes* in vitro. *(see test for explanation)*

	IN VIVO TREATMENT				
	0	**TiO_2**	**quartz**	**zymosan**	**PMA**
Mean	109	112	106	111	109
SD	19	22	12	20	12

The triggers used were 0, TiO_2, quartz, zymosan and PMA. Results are presented as percentage of medium control obtained in 1-3 separate experiments (3 wells/sample). Since no differences were obtained with any of the triggers, all of the data (both with and without triggers) has been pooled to derive the means as shown. The incubation time of the assay, however, is only 4 hours which might have been too short for the stimulatory effect of the particles to become apparent. The bronchoalveolar leukocytes were therefore cultured for three days *in vitro*, in the presence of particles, before proteolytic activity (measured as fibronectin degradation) in the culture supernatants was assessed. These experiments also showed no increased proteolytic activity in response to *in vitro* triggering (Table 12).

Table 12. The effect of in vitro *triggering over a 3 day period on the proteolytic activity of supernatants from control leukocytes and those obtained 5 days after intratracheal injection of particles. (see text for explanation)*

	IN VIVO TREATMENT (5 DAYS)			
	0	TiO_2	quartz	zymosan
mean	91	104	140	153
SD	10	3	13	6

Results are presented as percentage of control supernatant activity and are the mean of 1-3 separate experiments with 3 wells per treatment group. The *in vitro* triggers used were TiO_2, quartz, zymosan and PMA. Since the triggers had no effect, all of the data (both with and without triggers) for each leukocyte population was pooled to give an indication of overall differences in the proteolytic activity of different leukocyte supernatants. Although *in vitro* triggering did not enhance the proteolytic activity of inflammatory leukocyte supernatants, they had greater baseline activity than controls

3.5.5. Summary

The foregoing results indicate that different particles have varying potentials to elicit leukocyte recruitment when they first deposit in the lung. The persistence of the tissue response varies with the type of particle and is related both to the durability of the particles and to their ability to retain inflammogenic activity in the lung. Inflammatory bronchoalveolar leukocytes have enhanced proteolytic activity compared with resident alveolar leukocytes. Activation of the leukocytes is not mediated solely by exposure to particles *in vitro* but is dependent on *in vivo* exposure.

3.6. Bronchoalveolar leukocyte response in rats inhaling coalmine dust

Having established that the inflammatory response to intratracheal injection of particles was a sensitive indicator of the comparative harmfulness of particles gaining access to the lung, the next question addressed was how the alveolitis would develop in response to particles deposited in the lung by the more realistic inhalation mode, where the accumulation of a particle burden is more gradual than with intratracheal exposure.

In light of the progressive nature of pneumoconiosis, a further facet of this part of the study was to investigate the persistence of the alveolitis following cessation of dust

exposure. The cellular source of the proteinase activity was also investigated by assessing the inhibitor profile of the leukocytes. Previous experiments had indicated that, *in vitro*, the particles themselves did not alter the proteolytic activity of leukocytes (3.5). The effect of other triggers of leukocyte activation were therefore assessed, in order to determine whether exposure to coalmine dust would potentiate the response of leukocytes to additional stimuli which might be present in the inflammatory milieu of the lung.

3.6.1. Total number of leukocytes retrieved by bronchoalveolar lavage

The total number of leukocytes in the bronchoalveolar lavage increased with duration of dust inhalation (Figure 20); following 52 days of exposure there were significantly more (13.2[0.9]) (mean[sem])x10^6 leukocytes in the bronchoalveolar lavage of treated rats than in the controls (4.3[0.8]) ($p<0.001$).

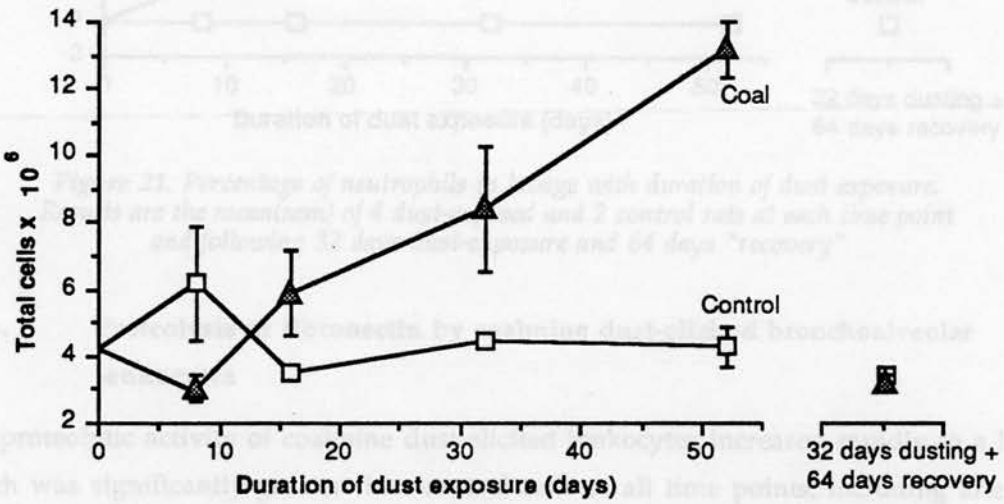

Figure 20. Total leukocytes in bronchoalveolar lavage fluid with time of dust exposure. Results are the mean(sem) of 4 dust-exposed and two control rats at each time point during dust-exposure and following 32 days dust-exposure and 64 days "recovery"

In those animals which were exposed to coalmine dust for 32 days followed by a period of 64 days breathing room air (recovery animals), the total number of leukocytes in the bronchoalveolar lavage returned to control levels.

3.6.2. Percentage of neutrophils in the bronchoalveolar lavage

There were no neutrophils in the bronchoalveolar lavage of control animals but increasing numbers were present in the treated animals as the duration of exposure increased (Figure 21); the increase was statistically significant at all time points ($p<0.001$).

Although the total number of leukocytes in the bronchoalveolar lavage of the recovery animals was similar to that of the controls (Figure 20), the percentage of neutrophils remained elevated. This is indicative that some degree of inflammation persisted long after cessation of dust exposure.

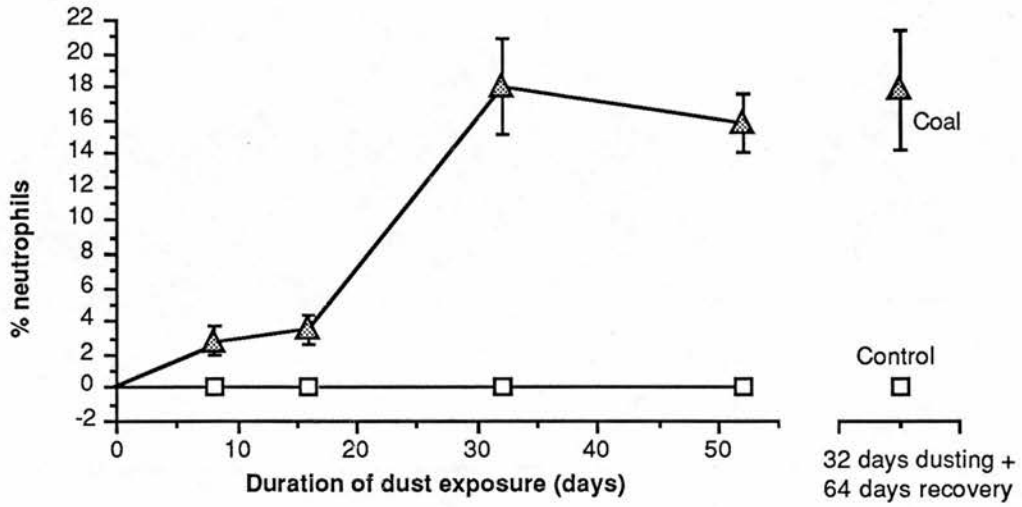

Figure 21. Percentage of neutrophils in lavage with duration of dust exposure. Results are the mean(sem) of 4 dust-exposed and 2 control rats at each time point and following 32 days dust-exposure and 64 days "recovery"

3.6.3. Proteolysis of fibronectin by coalmine dust-elicited bronchoalveolar leukocytes

The proteolytic activity of coalmine dust-elicited leukocytes increased rapidly to a level which was significantly greater than control cells at all time points, including after 64 days recovery ($p<0.001$) (Figure 22) but it did not increase with duration of dust exposure.

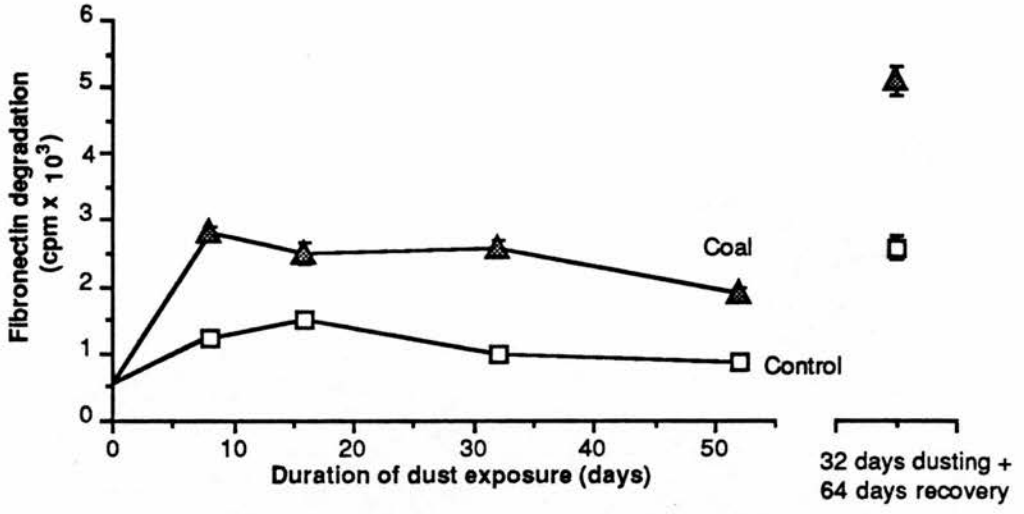

Figure 22. Activity of bronchoalveolar leukocytes in degrading fibronectin with duration of dust exposure, expressed as counts per minute of degraded fibronectin released into the supernatant medium/1x10^5 cells and are the mean(sem) of three wells per treatment group. Results are the mean(sem) of 4 dust-exposed and 2 control rats at each time point and following 32 days dust-exposure and 64 days "recovery"

The proteolytic activity of the bronchoalveolar leukocytes, averaged over all time points was 2983(562) (mean[sem])cpm for the dust-elicited cells and 1448(313) for the controls. To obtain an approximation of the potential protease burden in the alveolar region, the figures for total leukocytes in lavage and proteolytic activity per cell were multiplied (Figure 23).

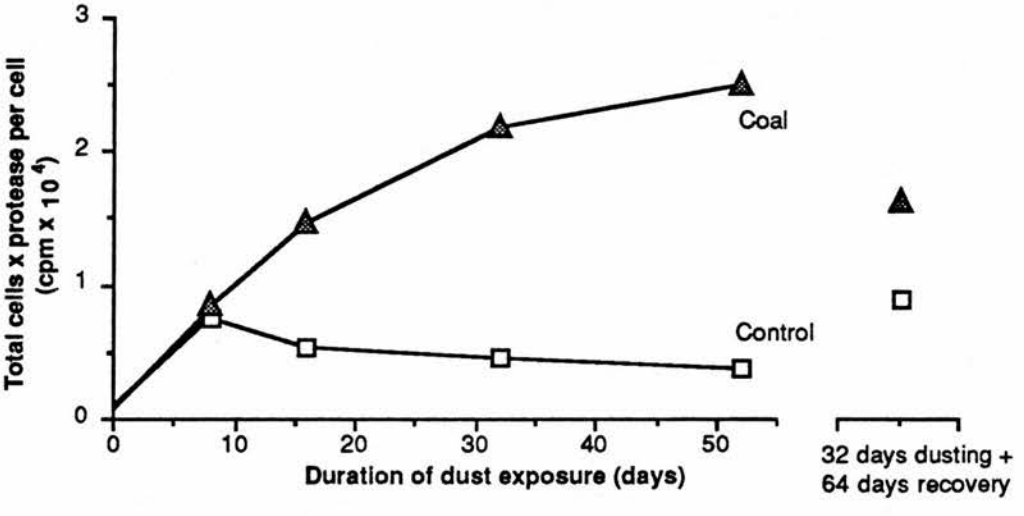

Figure 23. Proteolytic activity per cell multiplied by the total number of leukocytes in lavage and plotted against duration of dust exposure to estimate the potential burden of proteolytic activity in the alveolar region of the lung in arbitrary units. Results are the mean(sem) of 4 dust-exposed rats and 2 controls at each time point and following 32 days dust-exposure and 64 days "recovery

The total potential protease burden in the lung in arbitrary units, was 6-fold greater in the coalmine dust-exposed animals than in the controls at day 52 of exposure and still about double that of the controls in the recovery animals. These results also reflect ongoing inflammation in the alveolar region, long after the cessation of dust exposure.

3.6.4. Inhibitor profile of the proteolytic activity of coalmine dust-elicited bronchoalveolar leukocytes

To further investigate the cellular source of the increased proteinase activity, the proteolytic activity of the bronchoalveolar leukocytes 8 days after the start of dust-exposure was tested in the presence of inhibitors of serine proteinases (neutrophil) and metalloproteinases (macrophage) which were added into the assay together with the leukocytes. The inhibitors used were α1-PI, α2M, foetal calf serum and EDTA. Of these, only α1-PI is specific for serine proteinases whilst EDTA is specific for metalloproteinases; α2M and foetal calf serum inhibit both types of proteinase. Each inhibitor caused a dose-dependant inhibition of proteolysis (Figure 24) which was significant at 0.01, 0.1 and 1.0 mg/ml for a1-PI ($p<0.001$), at 0.5 mg/ml for α2M ($p<0.005$),at 1,5 and 10% serum ($p<0.005$) and at 1.0 mg/ml EDTA ($p<0.001$), thus confirming that matrix degradation was proteinase-mediated.

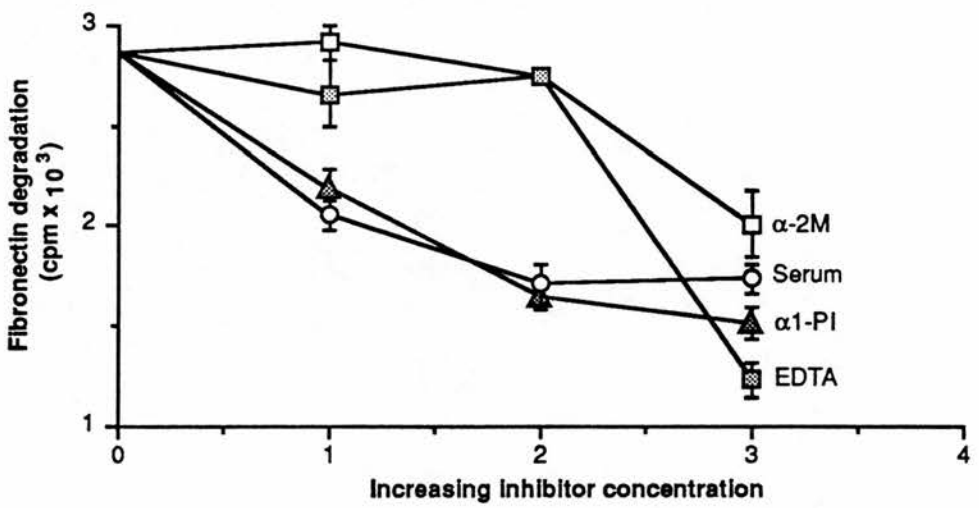

Figure 24. Inhibition of activity in the fibronectin proteolysis assay, of bronchoalveolar leukocytes from control rats or rats exposed to coalmine dust for 8 days. The inhibitor concentrations used were α1-PI and EDTA- 0.01, 0.1 and 1mg/ml, α2M- 0.005, 0.05 and 0.5mg/ml and serum- 1, 5 and 10%. Results are expressed as counts per minute of degraded fibronectin released into the supernatant medium and are the mean(sem) of three wells for each inhibitor concentration.

Previous experiments using exogenous enzymes (3.1.1.2.) indicated that EDTA and α1-PI could distinguish serine and metalloproteinases and should therefore distinguish macrophage from neutrophil proteinase activity. With the coalmine dust-elicited

leukocytes, α1-PI was a highly efficient inhibitor of the proteolytic activity of the leukocytes but EDTA had very little effect. The results suggest, therefore, that the proteolytic activity of the leukocytes was largely mediated by serine proteinase and hence was neutrophil-derived.

3.6.5. *In vitro* stimulation of the proteolytic activity of bronchoalveolar leukocytes

As previously demonstrated for other bronchoalveolar leukocyte populations (3.3, 3.5.1), neither the control nor the coalmine dust-elicited inflammatory leukocytes were stimulated by triggering *in vitro*; PMA and zymosan failed to elicit enhanced proteolysis of fibronectin by either population (Figure 25).

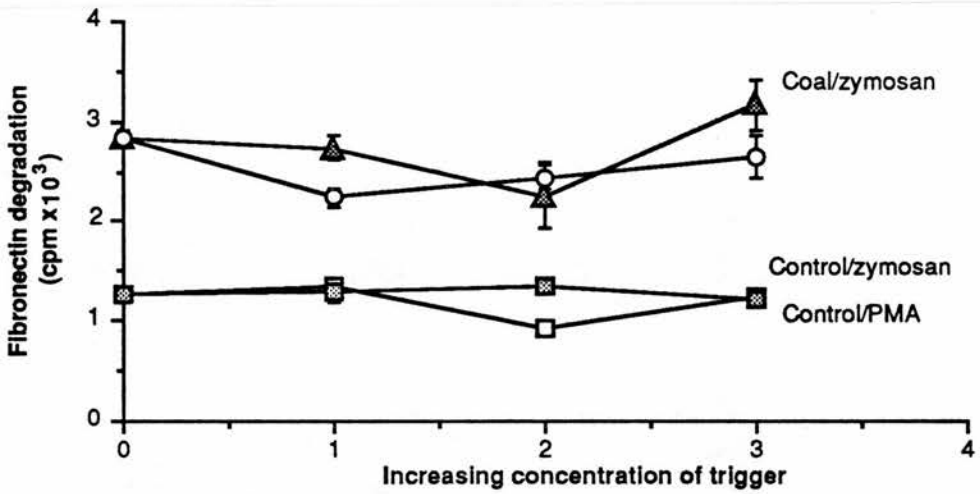

Figure 25. Triggering of the proteolytic activity of 8 day dust-elicited bronchoalveolar leukocytes and control cells by zymosan at 1, 10 and 100μg/ml and PMA at 0.1, 1.0 and 10 μg/ml. Results are expressed as counts per minute of degraded fibronectin released into the supernatant medium and are the mean(sem) of triplicate wells per concentration of trigger.

The failure of the coalmine-dust-elicited leukocytes to respond to further triggering *in vitro* may have been because they were already maximally stimulated *in vivo*; their proteolytic activity reached its maximum within 8 days from the start of dust exposure and remained constant thereafter. However, the evidence with other bronchoalveolar leukocyte populations suggests that leukocytes from the bronchoalveolar region of the rat are generally refractory to *in vitro* stimulation from a single stimulus at least.

3.6.6. Summary

Inhalation of coalmine dust, at plausible levels in relation to the industrial situation, evoked the gradual development of an alveolitis which increased with duration of

exposure. The inflammatory leukocytes had greater proteolytic activity than control leukocytes but an early increase in proteinase activity remained constant and was not time-dependent. The inhibitor profile of the inflammatory leukocytes at the 8 day time point suggested that their proteolytic activity was largely mediated by serine and hence neutrophil proteinases. Neither controls nor inflammatory leukocytes obtained following 8 days of dust exposure responded to *in vitro* triggering with PMA or zymosan which indicates, as before (3.3) that multiple *in vivo* stimuli are probably essential for leukocyte activation to take place.

3.7. Modulation of quartz-induced alveolitis by aluminium lactate

The fibrogenic potential of mixed dusts containing quartz is often less than predicted on the basis of the proportion of quartz in the dust. In mixed dusts whose fibrogenicity is less than would be predicted by the quartz content, it is possible that surface modification of the quartz particles by soluble compounds released by the non-quartz component(s) has rendered them less harmful. Minerals such as mica and smectite are present in some mixed dusts such as coalmine dust and can release aluminium compounds which may coat the quartz particles and so render them less toxic. This situation has been modelled *in vitro* by (a) precoating quartz particles with an aluminium compound and then comparing their inflammogenic potential with that of untreated quartz particles and (b) exposing rats to quartz and then administering aluminium lactate into the lung one month later and assessing the progression of the alveolitis.

3.7.1. Total leukocytes in the bronchoalveolar lavage

Intratracheal instillation of quartz evoked a marked and persistent alveolitis which was sustained up to 12 weeks after dosing (Figure 26); >90% of the bronchoalveolar leukocytes were viable.

Total leukocytes in lavage of the quartz-treated animals was significantly greater than controls at all time points ($p<0.001$). In animals dosed with quartz/aluminium lactate, there was a small increase in the number of leukocytes lavaged one week after treatment (8.6 [2.0] (mean[sem]) x 10^6) but this was not significantly greater than in the control bronchoalveolar lavage (3.7 (0.4)) and returned to control levels thereafter.

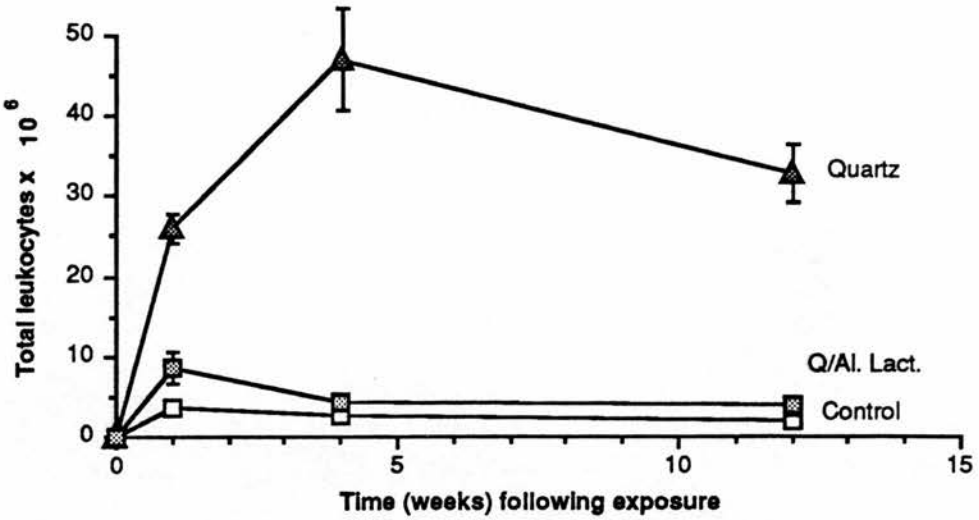

Figure 26. Total bronchoalveolar leukocytes in lavage with time following intratracheal injection of quartz or quartz pretreated with aluminium lactate. Q/Al. Lact.= quartz pretreated with aluminium lactate. Results are the mean(sem) of three rats per group in two separate experiments.

3.7.2. Percentage of neutrophils in the bronchoalveolar lavage

The percentage of neutrophils rapidly increased in the animals dosed with native quartz and remained at around 50% for up to 12 weeks (Figure 27).

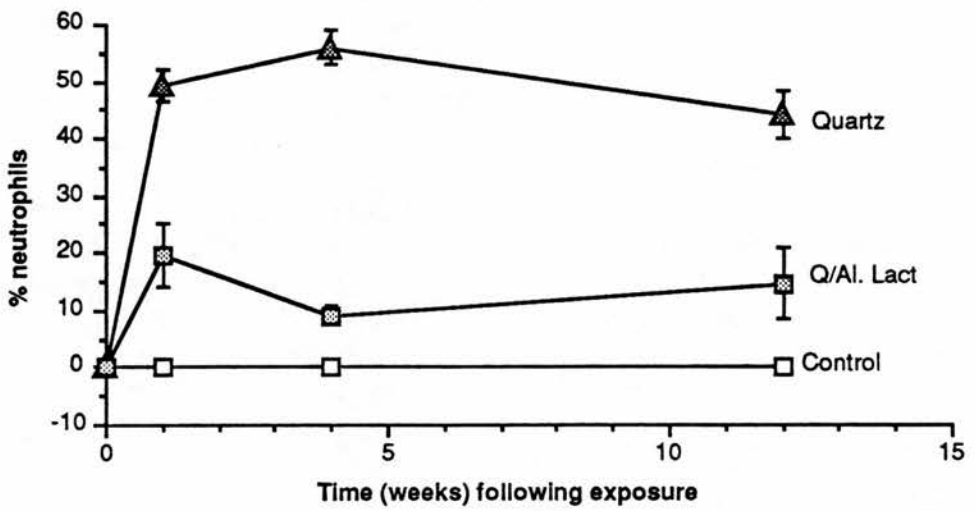

Figure 27. Percentage of neutrophils in the bronchoalveolar leukocyte population with time following intratracheal injection of quartz or quartz pretreated with aluminium lactate (Q/Al. Lact.). Results are the mean(sem) of three rats per treatment group in two separate experiments.

The inflammation was also sustained in the quartz/aluminium lactate-dosed animals. Although the percentage of neutrophils was less than in those treated with native quartz, there remained significantly more neutrophils than in the control animals up to 12 weeks after injection.

3.7.3. Proteolytic activity of the bronchoalveolar leukocytes

Bronchoalveolar leukocytes from quartz-dosed animals had greater ability to degrade fibronectin than control leukocytes (significant at 1 and 4 weeks, $p<0.001$, $p<0.005$ respectively) or those obtained from rats dosed with quartz/aluminium lactate (significant at 4 weeks, $p<0.05$) (Figure 28).

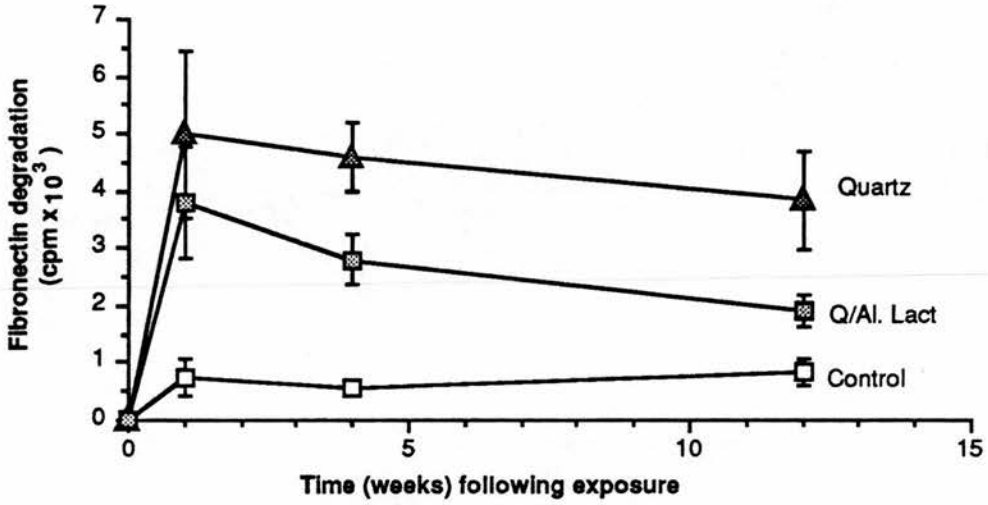

Figure 28. Fibronectin degrading activity of control bronchoalveolar leukocytes or those from rats exposed to quartz or quartz/aluminium lactate (Q/Al. Lact.) Results are expressed as counts per minute of degraded fibronectin released into the supernatant medium and are the mean(sem) of triplicate wells for three rats per treatment group in two separate experiments.

The latter were also more active than control leukocytes (significant at 4 weeks, $p<0.005$). The proteolytic activity of both the quartz-treated and the quartz/aluminium lactate-treated animals was maximal one week after treatment and declined linearly thereafter. The proteolytic activity per cell of the leukocytes was multiplied by the total number of leukocytes in the lavage to assess the potential proteinase burden in the lungs (Figure 29).

Although the activity per cell of the quartz-elicited leukocytes was less than double that of those elicited by quartz/aluminium lactate, the potential proteinase burden in the alveolar region was markedly different. In the former, there was a 13-fold increase over the controls at 12 weeks but in the latter, the increase was only 4-fold, so the proteolytic threat to the lungs was markedly reduced by precoating the quartz particles.

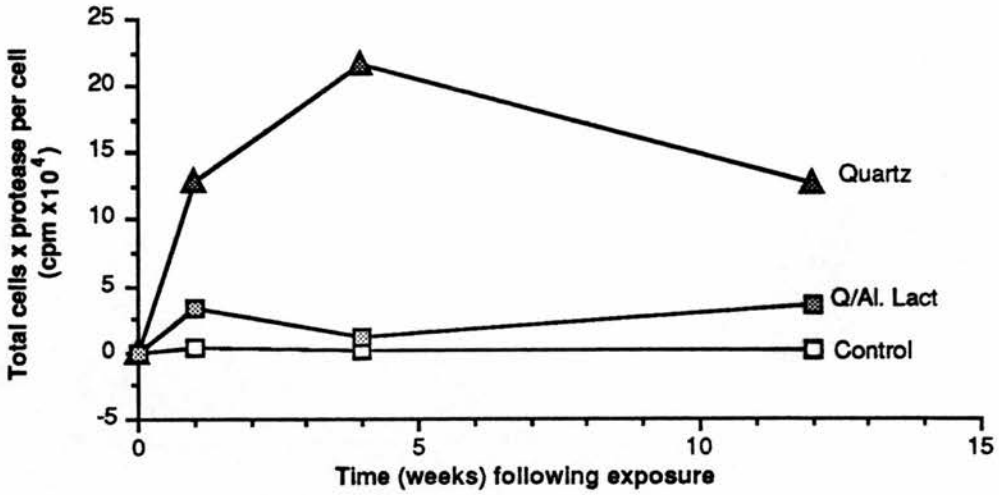

Figure 29. A measure of the potential proteinase burden of the whole lung in control rats and rats exposed to quartz or quartz/aluminium lactate(Q/Al. Lact.). Overall proteinase burden calculated in arbitrary units as total cell number in the bronchoalveolar lavage x proteolytic activity per cell. Results are the mean(sem) of triplicate wells per sample and three rats per treatment group in two separate experiments.

3.7.4. Delayed treatment with aluminium lactate

3.7.4.1 Total leukocytes in the lavage

In terms of the total number of leukocytes in the lavage, animals dosed with aluminium lactate 1 month after quartz exposure showed no reduction in alveolitis by one week after aluminium treatment (Figure 30).

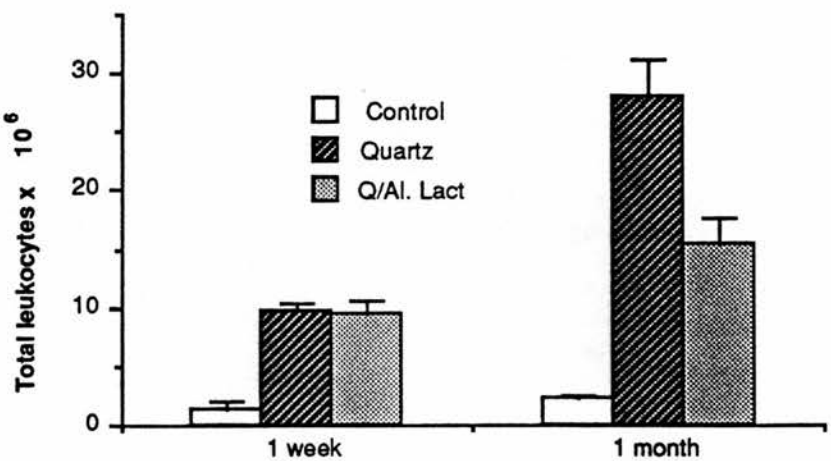

Figure 30. Effect of delayed treatment with aluminium lactate on the total number of leukocytes in bronchoalveolar lavage: 50μg aluminium lactate was injected intratracheally, 1 month following intratracheal injection of 1mg quartz and the lavage cell numbers were estimated 1 week or 1 month thereafter. Results are the mean(sem) of three rats per treatment group in two separate experiments.

There were 9.7(0.7) x 10^6 (mean[sem]) leukocytes in the quartz-treated animals and 9.4(1.2) in the quartz/aluminium-lactate-treated animals. By one month after aluminium lactate treatment, however, the total number of bronchoalveolar leukocytes had increased in both groups but there were significantly fewer leukocytes in aluminium-treated rats- 15.3(2.2) than in the untreated quartz animals- 28.0(7.8), ($p<0.001$). This suggests that aluminium treatment of quartz-exposed lung suppressed leukocyte recruitment.

3.7.4.2. Percentage of neutrophils in the lavage

Delayed aluminium lactate treatment also had an effect on the percentage of neutrophils in lavage (Figure 31).

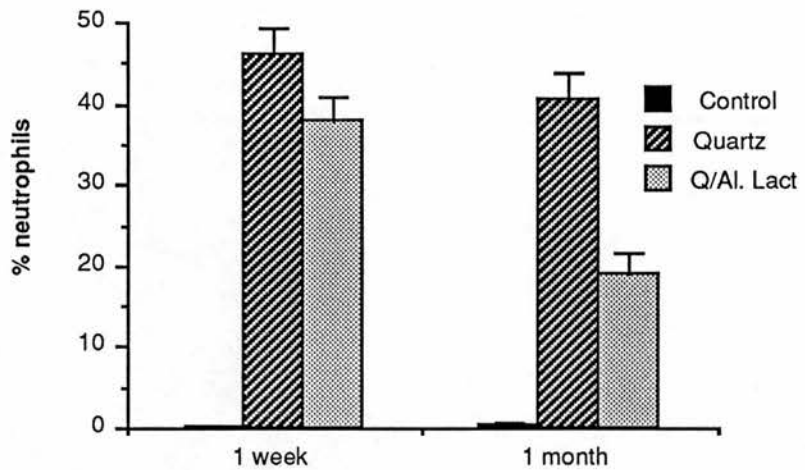

Figure 31. Effect of delayed treatment with aluminium lactate on the percentage of neutrophils in bronchoalveolar leukocytes from rats exposed to quartz for 1 month, followed by aluminium lactate for a further 1 week or 1 month. Results are the mean(sem) of three rats per group in two separate experiments.

One week after aluminium treatment there were fewer neutrophils in the lavage 38 (2.9)% (mean[sem]) than in the untreated quartz-dosed animals-46 (3.2) but the difference was not statistically significant. By one month after aluminium treatment, however, there was a significant reduction in the percentage of neutrophils in lavage 19(3.6) compared with the quartz animals 40.5(3.4) ($p<0.0025$).

3.7.4.3. The effect of delayed aluminium lactate in reducing macrophage and neutrophil recruitment to the bronchoalveolar region of the lungs.

The reduced influx of leukocytes, 4 weeks after aluminium lactate treatment could have been due to suppression of either or both macrophage and neutrophil recruitment. The absolute number of macrophages and neutrophils in lavage at 1 and 4 weeks were therefore

calculated from the data of total cells and percentage of neutrophils in lavage (Table 13). The percentage increase of each cell type was then calculated and revealed that recruitment of macrophages and neutrophils were both suppressed, neutrophils marginally more so than macrophages.

Table 13. Reduction of macrophage and neutrophil recruitment by aluminium lactate treatment of rats exposed to quartz.

	TREATMENT GROUPS			
	Total neutrophils		Total macrophages	
Weeks	Q	QA	Q	QA
1	4.5	3.6	5.8	5.2
4	11.2	5.8	16.8	9.5
% increase	267	161	290	183

Q= animals treated with quartz alone. QA= animals treated with quartz followed by aluminium lactate.

Results are presented as total cells of each type x 10^6 and % increase in total cells between 1 and 4 weeks.

3.7.4.4. Proteolytic activity of bronchoalveolar leukocytes following delayed treatment with aluminium lactate

Delayed aluminium lactate treatment of rats exposed to quartz had no effect on the proteolytic activity of the bronchoalveolar leukocytes either 1 week or 1 month after the aluminium lactate treatment (Table 14). Leukocytes from quartz-exposed and quartz / aluminium lactate-exposed rats were similar in their ability to degrade fibronectin and were significantly more active in this respect than control leukocytes ($p<0.005$).

Table 14 Effect of delayed aluminium lactate treatment on the proteolytic activity of quartz-elicited bronchoalveolar leukocytes

	FIBRONECTIN DEGRADATION	
	1 week	1 month
CONTROL	431(34)	531(169)
QUARTZ ALONE	2072(59)	2510(591)
QUARTZ/ALUMINIUM LACTATE	2595(76)	2316(613)

Results are expressed as mean(SEM) counts per minute of degraded[125I] fibronectin released for triplicate samples and two experiments at each time point.

3.7.5. Effect of aluminium lactate treatment on the inflammatory response to *C. parvum*

In order to ensure that the effect of delayed aluminium lactate treatment was specific for the quartz particles and not a generalised effect in inhibiting leukocyte responses, an alternative inflammogenic particle (*C.parvum*) was administered to the lungs and the effect of delayed aluminium lactate treatment on the inflammatory response to those particles was assessed. Lungs of animals dosed with *C. parvum* alone or *C.parvum* and concomitantly administered aluminium lactate were equally effective in mounting an inflammatory response (Table 15). Aluminium lactate had no effect on total leukocytes or percentage of neutrophils in the bronchoalveolar lavage and the proteolytic activity of the bronchoalveolar leukocytes also remained unaltered. This indicated that the effect of aluminium was indeed specific for quartz.

Table 15 Effect of aluminium lactate on the inflammatory response elicited by intratracheally instilled C.parvum.

		Days following intratracheal injection					
		1		3		15	
		Cp.	Cp/Al.	Cp.	Cp/Al.	Cp.	Cp/Al.
Total cells ($x10^6$)	$\bar{x}$	21.9	21.2	13.4	22.6	11.1	13.0
	sd	2.6	2.1	3.0	0.6	1.8	2.9
% PMN	$\bar{x}$	76	82	34	37	0.3	0.3
	sd	3.5	0.3	4.1	7.6	0.3	0.3
Proteolysis ratio		5.0	4.8	3.5	3.2	1.3	1.3

Cp=C.parvum; Cp/Al=C.parvum administered concomitantly with aluminium lactate.

Results represent the mean (SEM) for triplicate samples at each time point, except where the proteolytic activity is expressed as released counts as a ratio of background.

3.7.6. The tissue response to quartz and to quartz/aluminium lactate

One week after quartz or quartz/aluminium lactate treatment there was little evidence of tissue damage but occasional macrophage accumulations were present at bifurcations of alveolar ducts. By 1 month after quartz treatment, areas of alveolar lipoproteinosis were seen in conjunction with septal hyperplasia; these areas were minimal in animals treated with quartz/aluminium lactate. After 3 months, the tissue injury had progressed in both

treatment groups with the greatest damage apparent in the quartz-treated animals (Figure 32).

Rats treated with quartz/aluminium lactate showed marked areas of tissue injury but these were less severe than in animals dosed with quartz alone and comprised only about 5% of the entire tissue, compared with around 80% in the quartz-treated animals. Late dosing with aluminium lactate had no effect in reducing the tissue response to quartz.

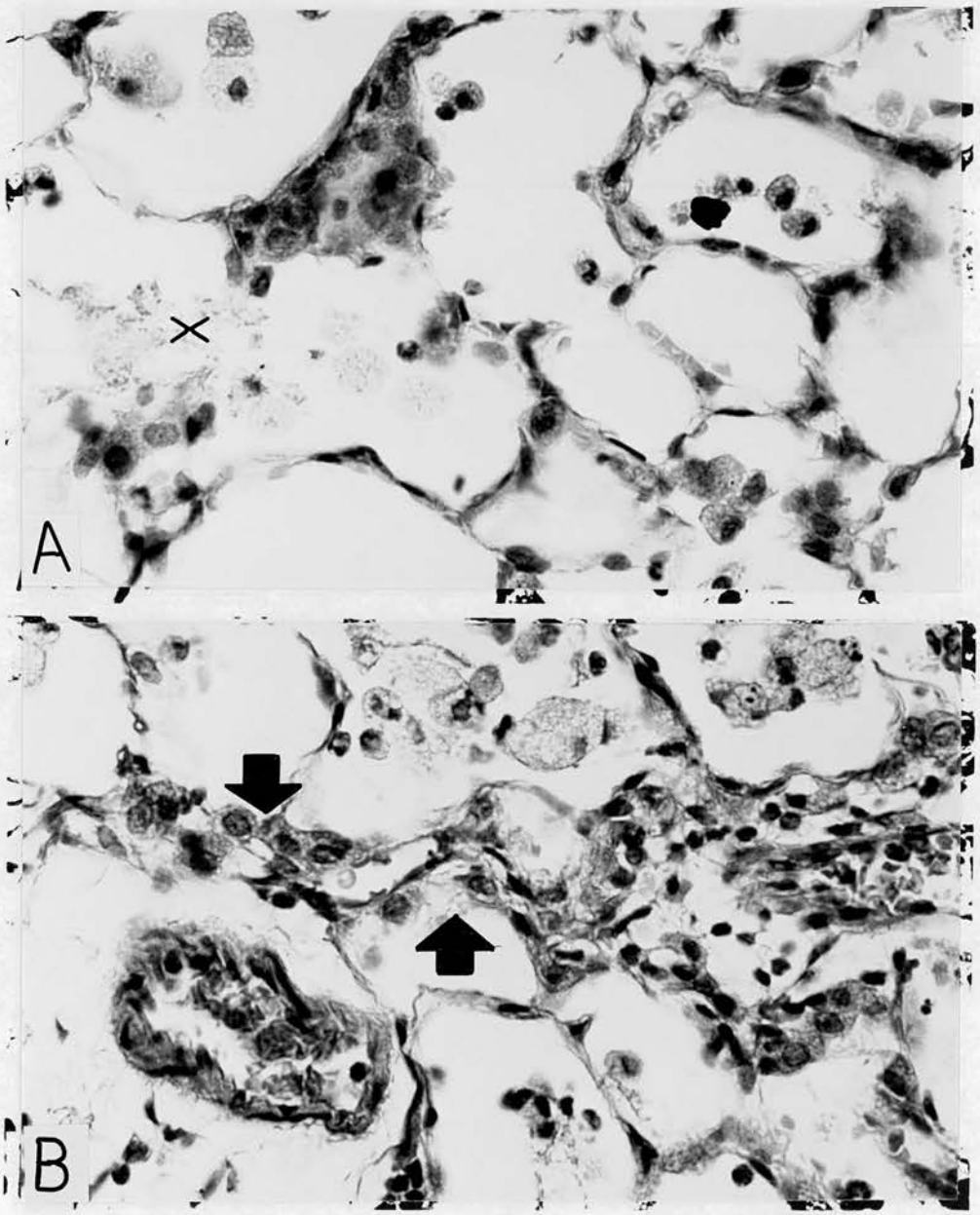

Figure 32. Tissue response 3 months after intratracheal injection of (a) 1mg quartz pretreated with aluminium lactate or (b) native quartz. (x500 magnification). Areas of hyperplasia (arrowed) were more extensive in lungs treated with quartz alone than in those treated with Q/Al.Lact. Lipoproteinosis (indicated by X in 8a was also seen extensively in quartz-treated lungs but does not show up in figure 8b.

3.7.7. Summary

The activity of quartz in biological systems has been related to its surface reactivity. This section of the thesis has addressed the role of quartz surface reactivity in mediating the inflammogenic potential of quartz. Aluminium is known to react with the surface of quartz particles and in this series of experiments a soluble aluminium compound, aluminium lactate reduced all of the measured parameters of the inflammatory response to quartz in rat lungs. This was true whether the aluminium was reacted with the quartz prior to- or administered after quartz injection. The aluminium acted on the quartz particles and not on the bronchoalveolar leukocytes as shown by its failure to reduce the inflammatory response to *C.parvum*. These results may go some way to explain the unexpectedly low toxicity of some mixed dusts containing both high levels of quartz and aluminium-containing minerals.

DISCUSSION

4. DISCUSSION

Alveolitis is a characteristic feature of the pneumoconioses (Voisin et al, 1985; Begin et al, 1986) and leukocyte neutral proteinases, which are elevated in the bronchoalveolar lavage of pneumoconiotic patients (Hayem et al, 1980; Sablonniere et al, 1983), have been implicated in the pathogenesis of these diseases. Many previous experimental studies have demonstrated the development of alveolitis in animals exposed to mineral dusts (see 1.10). In one of those studies (Sykes et al, 1983), levels of elastase in the bronchoalveolar lavage fluid were measured and were found to be increased in silica-exposed animals. Silica exposure *in vitro* has also been shown to cause increased elastase release by alveolar macrophages (Gulyas et al, 1988). However, there has been no systematic study of the role of leukocyte proteinases in the pathogenesis of pneumoconiosis. The aim of the work presented in this thesis was therefore to assess the development of alveolitis in rats exposed to mineral dusts and to measure the proteolytic activity of the inflammatory leukocytes. The specific aims were to develop a proteolysis assay which would effectively assess the proteolytic activity of bronchoalveolar leukocytes and then, using that assay, to measure the activity of leukocytes at various stages of the inflammation elicited in rat lungs by deposition of different types of dust particle.

4.1. The proteolysis assay

4.1.1. Leukocyte proteinases

Much of the work investigating the proteolytic potential of bronchoalveolar leukocytes has been accomplished by measuring the secretion of specific proteinases by alveolar macrophages (White et al, 1981b; Hibbs et al, 1987) and peripheral blood neutrophils (Janoff et al, 1979; Henson and Johnston, 1987). The content of individual proteinases in lysed peripheral blood neutrophils (Baugh and Travis, 1976; Gardi and Lungarelli, 1986), neutrophils from purulent sputum (Twumasi and Liener, 1972), alveolar macrophages (Levine et al, 1976) and peripheral blood monocytes (Senior et al, 1982) has also been assessed. However, inflammatory leukocytes secrete an array of proteinases and measuring secretion of only one of those proteinases is likely to lead to an underestimation, or in some cases (Levine et al, 1976), an overestimation of the true proteolytic potential of the leukocytes. In this study, therefore, a solid phase assay measuring proteolysis of connective tissue components was used to measure the overall proteolytic activity of viable bronchoalveolar leukocytes, and hence to predict their potential to inflict proteolytic damage on the delicate tissues of the alveolar septum.

4.1.2. Fibronectin degradation as a measure of proteinase

The assay used was first described by Johnston and Varani (1981) and was subsequently modified by Campbell et al (1982) for estimating the proteolytic potential of peripheral blood neutrophils using [^{125}I]fibronectin as substrate. In the lung, leukocytes are in close contact with the connective tissue of the extracellular matrix; the solid phase proteolysis assay technique, where the leukocytes are cultured on a matrix of connective tissue molecules, reflects this situation as closely as is possible *in vitro*. In the present study, the proteolysis assay was developed using radio-iodinated connective tissue molecules (fibronectin, laminin and denatured collagen types I and IV) and tested using exogenous proteinases. All of the connective tissue molecules labelled with high specific activity and consistently formed uniform matrices in microtitre plates. All of the matrix components were readily degraded by the proteinases, thus confirming the suitability of the assay for measuring the ability of leukocyte proteinases to digest alveolar connective tissue. Further work to standardise the assay was carried out using the [^{125}I]fibronectin matrix and this established that the assay was capable of detecting, in a dose- dependent manner, the activity of a variety of neutral proteinases. Different classes of proteinase could be discriminated on the basis of their inhibition by specific proteinase inhibitors; the exogenous proteinases collagenase (metalloproteinase), and elastase (serine proteinase), were inhibitable in the assay by EDTA (metalloproteinase inhibitor) and by α1-PI (serine proteinase inhibitor) respectively. The [^{125}I]fibronectin proteolysis assay developed for this thesis can thus be seen to be a relevant, sensitive and useful assay of leukocyte proteinase.

4.2. The experimental models

4.2.1. Pathogenic effects of silica and coalmine dust in rat lungs

Deposition of silica or coalmine dust in the human lung results in the formation of areas of nodular and interstitial fibrosis, emphysema and, particularly in the case of silica, type II cell hyperplasia and alveolar lipoproteinosis (Morgan and Seaton, 1984). The lesions which arise following silica deposition in rats are similar to those in man: the rat model shows the development of fibrotic lesions and alveolar lipoproteinosis in the lung parenchyma (Martin et al, 1977; Reiser et al, 1983) and also gives rise to emphysema (Wright et al, 1988). Inhalation of coalmine dust in rats leads to the development of typical pneumoconiotic lesions (Robertson et al, 1988). The foregoing studies confirm that the rat is a suitable model for the study of the pathogenesis of pneumoconiosis in man.

4.2.2. Exposure techniques

In the present study, two different methods of particulate-exposure were used to elicit inflammatory bronchoalveolar leukocytes:- intratracheal instillation and inhalation. Each technique has its own inherent advantages and disadvantages and so the most appropriate exposure method was chosen in each part of the study according to the research question being asked. The advantages of inhalation exposure are that it is a more realistic mode of exposure than intratracheal instillation and the particles are more homogeneously distributed throughout the lung (Brain et al, 1976). The accumulation of particles inhaled into the lung is influenced by many factors, in particular their airborne mass concentration and mass median aerodynamic diameter (MMAD). The airborne mass concentration of dust used in the inhalation experiments was 10 mg/m^3 of respirable dust, (ie dust with a MMAD of 3-5 microns). This dust concentration is near the maximum permissible level in British collieries which is $7mg/m^3$ measured in the return roadway of the pit face. The exposure regimen used in this series of experiments was, therefore, plausible for a workplace exposure. The two major disadvantages of inhalation exposure are that it is expensive and time consuming to run the inhalation chambers for long enough to build up a substantial deposited mass of dust in the lung. Also, differences in deposition and clearance patterns of different particles preclude the use of this technique for direct comparison of the intrinsic inflammogenic properties of the particles. Intratracheal instillation of particles was frequently used in this study, partly as a means of obtaining large numbers of inflammatory leukocytes quickly without the expense of running exposure chambers. It was also used to directly compare the inflammogenicity of particles. The major disadvantage of intratracheal injection is that all of the particles are deposited in the deep lung where some of them would not normally deposit with inhalation exposure. However, the consistent deposition of a known mass of particles using the intratracheal technique, is of distinct benefit when comparing the intrinsic inflammogenic properties of different types of particle. In this study, therefore, intratracheal instillation was used to compare the magnitude and persistence of the inflammatory bronchoalveolar leukocyte response elicited by a variety of particles.

4.3. Assessment of the proteolytic activity of bronchoalveolar leukocytes

4.3.1. Proteolysis of laminin and denatured collagen types I and IV and dose and time-responses of fibronectin proteolysis

Preliminary work in measuring the proteolytic activity of bronchoalveolar leukocytes was carried out using resident alveolar macrophages and two inflammatory bronchoalveolar

leukocyte populations of varying macrophage and neutrophil content, to establish optimum conditions for the assay. The proteolytic activity of the bronchoalveolar leukocytes was both dose- and time-dependent and was inhibitable by antiproteinases but not antioxidants. This reinforced our earlier finding that oxidants do not play a major part in tissue injury in inflamed lung (Donaldson et al, 1988d) but is contrary to previous suggestions that leukocyte oxidants might contribute to matrix damage in inflamed lung (Riley and Kerr, 1985). These results confirmed that the assay could detect differences in the proteolytic activity of different bronchoalveolar leukocyte populations and established that matrix degradation by the leukocytes was proteinase-mediated as previously described (Chapman and Stone, 1984; Sibille et al, 1986). Each of the bronchoalveolar leukocyte populations degraded denatured type I and type IV collagen, fibronectin and laminin. Thus, both resident and exudate bronchoalveolar leukocytes have proteolytic activity which is capable of damaging several of the connective tissue molecules present in the alveolar septum. The low proteolytic activity of resident alveolar macrophages, found in this study, may be an artifact caused by limited activation of the cells during culture on the fibronectin matrix (Kuriowa et al, 1988). This level of activity has also been reported in bronchoalveolar lavage cells from healthy human volunteers (Chapman and Stone, 1984). However, the in vitro activity may actually reflect macrophage activity in vivo. This activity may be an indication of a potential proteinase burden in the alveolar region which can be effectively limited by the normal antiproteinase screen, so preventing connective tissue damage. The increased activity of the inflammatory leukocytes in this rat model of lung inflammation supports the current view (see 1.7.) that inflammatory leukocyte proteinases play a major part in the tissue damage associated with chronic lung inflammation.

4.3.2. Relationship between neutrophil content and proteolytic activity

Neutrophil proteinases have frequently been cited as being central to connective tissue proteolysis in inflamed lung (Spitznagel et al, 1980; Gadek et al, 1984; Janoff, 1985) because neutrophils, constitutively, contain considerably more neutral proteinase than macrophages (Janoff et al, 1979; Gadek et al, 1984). In the preliminary experiments in this study, therefore, the role of neutrophils in connective tissue proteolysis was addressed by measuring the proteolytic activity of bronchoalveolar leukocytes of varying neutrophil content. *C.parvum* leukocytes contained most neutrophils and were the most active, quartz leukocytes contained fewer neutrophils and were less active than the *C.parvum*-leukocytes but were more active than the resident macrophages (no neutrophils). Although there was a relationship between proteolytic activity and the proportion of neutrophils in each population, the correlation was not perfect with more proteolytic activity in the

inflammatory populations than would be predicted by their neutrophil content. This suggests that 1) inflammatory macrophages might also contribute to the increased proteolytic activity of the quartz leukocytes, or 2) neutrophils in an inflammatory population containing a high proportion of macrophages, had greater proteolytic activity than those in populations containing minimal numbers of macrophages. Further experiments were therefore carried out to compare the proteolytic activity of inflammatory macrophages with that of exudate neutrophils and resident macrophages; these are discussed later in this chapter (4.9).

4.3.3. Proteolytic activity of *C.parvum*-elicited cell supernatants, lysed cells and intact, viable cells

In order to investigate the mechanisms of connective tissue proteolysis by leukocytes, the cellular source of the proteolytic activity was assessed by comparing intact cells with lysed cells and cell supernatants. The proteolytic activity of viable *C.parvum*-elicited bronchoalveolar leukocytes was greater than that of lysed cells or cell supernatants. Supernatants of human peripheral blood neutrophils are also reported to be less active than intact cells in causing breakdown of fibronectin in amnionic membranes (Sibille et al, 1986). There are several aspects of leukocyte function which could account for the greater proteolytic activity of intact cells:- 1) loss of enzyme activity during production of supernatants or lysates, 2) denovo synthesis of enzymes by alveolar macrophages in the course of the assay, 3) acid proteinases such as cathepsin B (Chapman and Stone, 1984) which is associated with the cell membrane, may play a part in matrix proteolysis by intact cells and cell lysates but would not be active in cell supernatants, 4) viable cells may have ways to maximise their ability to degrade connective tissue molecules; intact cells may increase their proteolytic activity by localised sequestration of acid and/or neutral proteinases at the site of close contact between the cell and the matrix (Werb et al, 1980; Campbell et al, 1982). This could substantially increase their concentration at that point and therefore increase their proteolytic potential, 5) proteinase inhibitors such as α1-PI and α2M, secreted by alveolar macrophages, may interact more readily with proteinase in a cell-free system; the site of close contact between cells and matrix may exclude concomitantly secreted inhibitors (Campbell et al, 1982) which would be free to inhibit the proteinases in cell supernatants or lysates. However, inhibitor secretion by macrophages is unlikely to have a major effect on the proteolytic activity of the *C.parvum*-elicited leukocytes because macrophages comprise <15% of the leukocytes in that population. Further experiments are therefore warranted to investigate the mechanisms of increased matrix proteolysis by intact, viable leukocytes. A time course of the proteolytic activity of leukocyte supernatants during 4hr incubation at 37°C would

determine whether the proteinases lose activity during preparation of supernatants and the contribution of membrane-bound proteinases to that activity could be estimated by measuring proteolysis by isolated leukocyte membranes. The relative contribution of acid and neutral proteinases to matrix proteolysis could be compared by measuring the proteolytic activity of leukocyte supernatants at acid and neutral pH.

4.4. Inhibition profile of bronchoalveolar leukocytes

The lung parenchyma contains an antiproteinase screen which inactivates leukocyte proteinases and thus prevents excessive tissue degradation under normal circumstances (see 1.4.4.). In chronic inflammatory lung disease the antiproteinase screen may be overcome by the increased number and proteolytic activity of inflammatory leukocytes, so permitting excessive connective tissue proteolysis and leading to tissue derangements (see 1.7.7.). The inhibition profile of resident and inflammatory bronchoalveolar leukocyte populations in response to α1-PI and α2M, was therefore measured in order to assess their inhibitability by antiproteinases present in the alveolar region. The proteolytic activity of both the quartz and the *C.parvum* inflammatory leukocyte populations was significantly reduced by each inhibitor but there was no significant reduction of control cell activity. The lack of significant inhibition of the control cells probably reflects the low initial level of proteolysis, which was barely above background. In other experiments where the proteolytic activity of control leukocytes was greater, there was significant inhibition of proteolysis but some residual proteolytic activity was always retained. Similarly, although the proteolytic activity of the inflammatory bronchoalveolar leukocytes was significantly reduced by the antiproteinases, a portion of their activity was also retained; this suggests a possible mechanism whereby connective tissue damage may take place in the lung, even in the presence of antiproteinases.

4.4.1. Exclusion of antiproteinases from sites of close contact between cells and matrix

Large molecular weight inhibitors may be excluded from the site of close contact between leukocytes and the extracellular matrix (Werb et al, 1980; Johnson and Varani, 1981; Campbell et al, 1982; Chapman and Stone, 1984) and connective tissue proteolysis could therefore proceed unimpeded at this privileged site. To test this hypothesis, control leukocytes and inflammatory bronchoalveolar leukocytes elicited by TiO_2 or quartz treatment were allowed to preadhere to a fibronectin matrix for 30 minutes before the inhibitors were added to the wells. In wells where inhibitor and cells were added at the same time, there was significant inhibition of the proteolysis caused by all three leukocyte populations. However, no significant inhibition occurred in any of the wells

where the cells had been preincubated on, and allowed to adhere to the matrix. This indicates that inhibitors can indeed be excluded from the privileged site where cells and extracellular matrix components are in intimate contact.

4.4.2. Ineffectiveness of the antiproteinase screen of the lung

The non-inhibitable portion of the proteolytic activity of resident alveolar macrophages may contribute to the low-level turnover of connective tissue molecules in normal lungs (Senior and Campbell, 1983; Gadek et al, 1984). In inflamed lung, both the total number and proteolytic activity of leukocytes is greatly increased and gives rise to a consequently increased proteinase burden in the alveolar region. Proteolytic injury in inflamed lung has previously been thought to arise when the increased burden of secreted proteinases overloads the antiproteinase screen. The results presented here suggest that the non-inhibitable portion of leukocyte proteinase activity may also contribute to such injury.

4.4.3. Inhibition profile of leukocyte populations containing different proportions of macrophages and neutrophils

The inhibition profile of the three leukocyte populations was not what would be predicted from their neutrophil content. Macrophages secrete metalloproteinases (Banda and Werb, 1981; White et al, 1981b) and neutrophils secrete serine proteinases (Travis and Salvesen, 1983). Therefore, based on the experiments using exogenous proteinases, the *C.parvum* (>80% neutrophils) and quartz (around 50% neutrophils) populations should have responded differently to inhibition by the serine proteinase inhibitor, α1-PI. In fact, they were both equally inhibitable which suggested that the increased proteolytic activity of both inflammatory populations was due to serine proteinases. This result might be explained if the macrophages in the quartz population were in fact newly recruited peripheral blood monocytes; these have been shown to contain serine proteinase activity which is around 25% that of neutrophils (Senior and Campbell, 1983). In addition, macrophages can sequester and subsequently release active neutrophil elastase (McGowan et al,1984) and this may also contribute to the apparent serine proteinase activity of the quartz leukocytes.

4.5. The proteolytic activity of human bronchoalveolar leukocyte populations

4.5.1. Comparative activity of lung and peripheral blood leukocytes

Human leukocytes had proteolytic activity which varied depending on their source. Three homogenous (ie >90% pure) populations of leukocytes were used:- 1) peripheral blood

neutrophils, obtained from a healthy volunteer, 2) minced lung macrophages, prepared by mincing excised human lung tissue, obtained from a site distal to a tumour and 3) bronchoalveolar macrophages, obtained from the lungs of an ex-smoker suspected of having a concurrent respiratory tract infection. The peripheral blood neutrophils, and minced lung macrophages exhibited marked proteolytic activity which was dose and time dependent but bronchoalveolar macrophages showed very little activity.

4.5.2. Factors influencing the proteolytic activity of bronchoalveolar leukocytes

Previous work investigating the proteolytic activity of human leukocytes has demonstrated proteolytic activity in peripheral blood neutrophils which was increased in patients with lung disease (Burnett et al, 1987). Interstitial leukocytes have been previously shown to be functionally more active than alveolar leukocytes (Weissler et al, 1986) and this may account for some of the increased proteolytic activity of the minced lung preparation. However, cytokines such as IL-1 and TNF are likely to be present throughout tumour-bearing lungs and, through their actions in mediating leukocyte activation, may also account for the increased proteolytic activity of the minced lung leukocytes (Larrick and Kunkel, 1988). The low neutrophil count (1%) in the bronchoalveolar leukocyte population suggested that the lungs were not acutely inflamed and therefore the leukocyte population might be representative of resident alveolar macrophages. These results suggest that in normal human lungs the resident alveolar macrophages have little proteolytic activity. Bronchoalveolar macrophages from cigarette smokers (Hinman et al, 1980) and pneumoconiotics (Sablonniere et al, 1983), ie in situations where there is inflammation and therefore inflammatory mediators in the lung, have increased proteinase activity. This, and the finding of minimal proteolytic activity of the bronchoalveolar leukocytes in the present study, supports the conclusions that 1) the increased proteolytic activity of macrophages from the minced lung preparation was due to activation by inflammatory mediators in vivo in the diseased lungs and 2) resident bronchoalveolar macrophages have minimal proteolytic activity.

4.5.3. Effect of in vitro triggers on the proteolytic activity of human peripheral blood and bronchoalveolar leukocytes

The proteolytic activity of the human peripheral blood neutrophils was increased following triggering *in vivo* which may reflect their key role in acute inflammation and host defence. The activity of the bronchoalveolar macrophages, however, was unaltered. This may be indicative of the functional status of resident alveolar macrophages which is suppressive of immune responses (Holt,1986) and may also render the macrophages refractory to stimulation. However, alveolar macrophage responses to exogenous stimuli

are complex and some require only a single stimulus, eg exposing bronchoalveolar macrophages *in vitro* to quartz, causes increased production of IL-1 (Kusaka et al, in press). Activation of alveolar macrophages for increased proteinase secretion may require the synergistic action of several stimulatory factors such as IL-1, TNF and interferon gamma, which occur at inflammatory sites (Billingham, 1987) and are likely to be present in the milieu of inflamed lungs.

4.6. The relative contribution of inflammatory macrophages and neutrophils to proteolytic injury in inflamed lungs

All of the foregoing results have demonstrated that in inflamed lung, there are substantial increases in numbers of both macrophages and neutrophils. Many investigators propose that neutrophils are the primary source of proteinase capable of damaging tissue in inflamed lungs because of their large complement of proteinases (Spitznagel et al, 1980) and since neutrophil elastase readily degrades elastin, collagen, fibronectin and glycosaminoglycans (Senior and Campbell, 1983). Crystal et al (1984) regarded the neutrophil as being so important to the disease process that they classified the interstitial lung diseases as either neutrophilic or lymphocytic. However, macrophages are also capable of substantial proteolytic activity (see 1.7.4.) and may therefore play an active part in tissue proteolysis in inflamed lungs (Fels and Cohn, 1986). In chronic inflammatory lung disease, neutrophils frequently comprise only 5 - 10% of the inflammatory leukocyte population. So, even if their proteolytic potential is tenfold greater on a per cell basis than that of macrophages, as previously suggested (Janoff et al, 1979; Gadek et al, 1984), their proteolytic activity would not be greater than that of the remaining 90% of macrophages in the alveolar region. In addition, neutrophil serine proteinases are more readily inhibited than macrophage metalloproteinases by proteinase inhibitors such as α1-PI in the alveolar region. So macrophage proteinases may also play a key role in the development of proteolytic damage in chronically inflamed lung.

4.6.1. Proteolytic activity of neutrophils and resident and inflammatory macrophages from the bronchoalveolar region of the lung

To assess the relative proteolytic potential of inflammatory macrophages and neutrophils, cells were separated from a quartz-elicited bronchoalveolar leukocyte population that was approximately 50% macrophages and 50% neutrophils. The proteolytic activity of a homogeneous population of inflammatory macrophages elicited by intraperitoneal injection of tryptose phosphate broth was also assessed. All of the inflammatory macrophage populations had increased proteolytic activity compared with resident alveolar macrophages and exudate neutrophils had proteolytic activity which

was around 15-30% greater than that of the inflammatory macrophages. Thus, compared with resident alveolar macrophages, inflammatory lung macrophages do exhibit increased proteolytic activity. In inflamed lungs where their numbers often substantially outweigh those of neutrophils, macrophages may make a major contribution to proteolytic damage. This finding may be of importance in the development of intervention strategies for the prophylaxis and management of chronic inflammatory lung disease.

4.7. Comparative inflammogenic properties of particles deposited in the lung by intratracheal instillation

4.7.1. Factors influencing the pathogenic potential of particles depositing in the lung

Toxic products and growth factors are released by inflammatory leukocytes at sites of inflammation in the lungs and so "bystander injury" to the surrounding host tissue, leading to excessive tissue breakdown and/or mesenchymal overgrowth are likely to result. The major difference between lung disease which resolves without causing pathological change in the lung and pathogenic lung disease may be the magnitude and duration of the alveolitis and the proteolytic activity of the bronchoalveolar leukocytes (Fantone and Ward, 1984). In pneumoconiosis there are three key factors which could determine the persistence of lung inflammation in response to mineral dust:- 1) duration of inhalation exposure to particles, 2) deposition and retention of harmful particles within the alveolar region and 3) persistence of the ability of particles in the lung to impart harm to the tissue. The effects of duration of exposure to mineral dust in causing lung inflammation (Donaldson et al, 1988a) and interstitial fibrosis (Davis et al, 1978) have been addressed previously and these studies showed that both are dose- and time-related. The ability of particles to persist within lung tissue has been assessed by measuring clearance rates following inhalation exposure (Vincent and Donaldson, in press); that study showed little difference in clearance between harmful and non-harmful dusts. Thus the major descriptor of the intrinsic potential harmfulness of a dust appears to be the retention of its biological reactivity in the lung.

4.7.2. The influence of type of particle on the magnitude and persistence of their biological reactivity in the lung

The key parameters of the magnitude of the inflammogenic potential and persistence of biological reactivity of particles deposited in rat lungs were assessed in the present study, using particles of different durability and pathogenic potential. Comparisons were made of the inflammatory response following a single intratracheal injection of the non-durable,

biological particulates, *C.parvum* (a heat-killed bacterial preparation) and zymosan (an extract of yeast cell walls), the inert dust, TiO_2 and the pathogenic dust, quartz; the latter two are non-biodegradable particles. TiO_2 is a particulate that is widely used in the food and paint industries but is not associated with pathology in occupationally exposed groups (Chen and Fayerweather, 1988); it is commonly used as an "inert" control dust in experimental studies *in vitro* (Le Bouffant et al, 1980; Donaldson and Brown, 1988) and *in vivo* (Moores et al, 1980; Donaldson et al, 1988a). By contrast, occupational exposure to quartz is associated with development of the fibrotic lung disease, silicosis (Davis, 1986) and quartz has been shown to have potent cytotoxic effects *in vitro* (Donaldson and Brown, 1988) and marked inflammogenic potential (Donaldson et al, 1988a).

4.7.3. Assessment of the magnitude and persistence of biological reactivity of different particles deposited in the lung

The results show that deposition of particulate matter in the lung evokes an inflammatory response manifest as increases in the total number of lavageable leukocytes and the proportion of neutrophils in the lavage population. This is true whether the particulate is a typical biological inflammogen eg *C.parvum* or zymosan, a harmful mineral dust - quartz, or an inert dust - TiO_2. In contrast with the report by Shen et al (1988), the pathogenic potential of particles was found to be independent of the magnitude of the initial inflammatory response which they evoked. *C.parvum*, which does not cause pathological change, following a single deposition in the lung caused the greatest leukocyte recruitment at 1 day whilst quartz, which has marked pathological potential, caused less leukocyte recruitment and was similar in activity to zymosan at that timepoint. However, most strikingly, the persistence of the inflammatory response was related to the pathogenic potential of the particles. Inflammation was rapidly resolved with the two biological particles and TiO_2 but the pathogenic dust, quartz, evoked a sustained alveolitis. The initial leukocyte recruitment is likely to be related to secretion of leukocyte chemotaxins following phagocytosis of particles by the resident alveolar macrophages (Lugano et al, 1982) and may also be related to direct complement activation by interaction of particles with the alveolar lining fluid (Donaldson et al, 1987; Warheit et al, 1988). The majority of particles depositing in the lung are no longer free to react extracellularly after 1-2 days, due to phagocytosis (Ferin, 1971) or extracellular transport to lymph nodes (Lehnert et al, 1988). The reduction in inflammation from 1 day onwards supports the theory that phagocytosis and complement activation by free particles may be an important mechanism in generating the acute inflammatory response. However, alveolar inflammation in response to quartz did not subside after 1 day but increased up to 30 days after injection when there were ten-fold more leukocytes in the bronchoalveolar

lavage of the quartz-exposed animals than in the controls, or in the other exposed groups. So, alternative explanations must be sought for the sustained inflammogenic properties of quartz.

4.7.4. Mechanisms of particle pathogenicity

A commonly invoked mechanism is that the pathogenic potential of a particle is related to its cytotoxicity (Bowden and Adamson, 1984) but recent evidence suggests that other mechanisms may also be involved in dust-induced inflammation in the lung. Quartz-elicited bronchoalveolar leukocytes in this study and in rats exposed to quartz by inhalation (Donaldson et al, 1988c) have been shown to be >99% viable and remained so during 24h culture *in vitro*. Studies exposing control rat alveolar macrophages *in vitro* to low levels of quartz also showed that the cells remained viable, although increasing the concentration of quartz did lead to cell death (Donaldson and Brown, 1988). The surface of quartz particles is highly charged and reacts with cell membranes by acting as an electron donor (Nolan et al, 1981). The charged surface of quartz may be the means whereby it kills cells at high concentrations but at low concentrations, where the macrophages remain viable, interaction of intracellular quartz with cell components may lead to macrophage activation with its attendant consequences for enhancing and prolonging the inflammatory response (Hamilton, 1980; Kusaka et al, in press). Further experiments have been carried out to investigate the role of the free quartz surface in eliciting and sustaining an inflammatory response and these are discussed in section 4.8.

4.7.5. Particle-elicited leukocyte proteinase burden in the alveolar space

Initially there were no differences in the proteolytic activity of leukocytes elicited by the different particles. However, in keeping with the sustained nature of the quartz alveolitis, the proteolytic activity of the quartz leukocytes also remained elevated; with the other three particles, the proteolytic activity of the leukocytes decreased as the alveolitis resolved. By integrating total leukocyte counts with proteolytic activity per cell, a measure of the potential proteinase burden in the alveolar region was obtained for each particulate. The difference between pathogenic and non-pathogenic particles was immediately apparent. The overall alveolar proteinase burden was greatest in the *C.parvum*-treated rats at 1 day but thereafter, only in the quartz-treated animals was there a sustained elevated proteinase burden which was 5-fold greater than in the controls. The lungs can therefore cope with a markedly elevated proteinase burden in an acute situation (eg 1 day *C.parvum*) but where there is persistence of a lower level of proteinase activity, then pathological change results. Thus the aspects of the biological reactivity of quartz which appear to be related to its pathogenic potential are the ability

to evoke and sustain alveolar inflammation and to thus maintain a persistent proteinase burden in the alveolar region.

4.7.6. *In vitro* triggering of control and inflammatory leukocytes

It is of interest that despite the marked inflammogenic potential of the biological and mineral dust particles following intratracheal injection, none had any effect in eliciting leukocyte activation *in vitro*. Even the quartz particles, which do have some ability to cause macrophage activation *in vitro* as assessed by IL-1 secretion (Kusaka et al, in press), had no ability to elicit increased proteolytic activity *in vitro*. There is ample evidence that leukocytes from other sites in the body eg the peritoneal cavity, secrete increased levels of proteinases in response to a phagocytic stimulus (Werb and Reynolds, 1974). This reinforces the theory that alveolar leukocytes are less readily stimulated and confirms the previous work with human bronchoalveolar leukocytes (4.5.). The effect of quartz particles on leukocytes may therefore be to prime them to respond to further signals in the milieu of the inflamed lung. This question could be addressed by investigating the proteolytic activity of leukocytes treated with quartz and inflammatory cytokines such as IL-1 and TNF.

4.8. Inflammogenic effects of inhaled coalmine dust

4.8.1. Lung responses to coalmine dust in experimental models

There have been many studies which have investigated the *in vitro* biological effects of coalmine dust (see 1.10.) but few which have assessed its *in vivo* effects. In the *in vivo* studies, the parameter most commonly studied has been the development of fibrotic lesions in the lungs (Le Bouffant et al, 1977); only one previous report has measured the inflammatory response to inhaled coalmine dust exposure (Bingham et al, 1977) and that study considered the role of acid proteinases in the disease process. In the present study, the role of neutral proteinases in the development of pneumoconiosis was addressed by measuring the inflammatory response in lungs of rats inhaling coalmine dust; the proteolytic activity of the bronchoalveolar leukocytes was then measured using the fibronectin proteolysis assay. Chronic inhalation exposure for up to 52 days, to low levels of coalmine dust, led to the gradual accumulation of inflammatory leukocytes in the alveolar region. These results are contrary to those of Bingham et al (1977) who reported no increase in total cells in the bronchoalveolar lavage of rats inhaling similar levels of coalmine dust. However, fundamental differences in the method of preparing the dust may explain these differences. The harmful minerals associated with the coalmining process are derived, in the main, from the roof and floor of the coal seam and would form a very

low part of the crushed coal sample used by Bingham et al (1977); crushed coal would also produce a finer dust that would be less likely to deposit in the alveolar region.

4.8.2. Persistence of the inflammatory effect of coalmine dust, post-exposure

In the present study, the total number of leukocytes in the alveolar region was elevated during dust exposure but returned to background levels during the 64 day post-exposure period. However, the percentage of neutrophils remained elevated which suggests that coalmine dust, like the quartz tested in the intratracheal study, has some ability to sustain an inflammatory response. The persistent inflammatory response elicited by coalmine dust, however, was less than that elicited by the more harmful dust, quartz. This reinforces the suggestion that dusts which have pathogenic potential may act by eliciting a sustained inflammatory response in the alveolar region and that response may be related to the harmfulness of the dust.

4.8.3. Proteolytic burden in the alveolar region of rats inhaling coalmine dust

The proteolytic activity of the inflammatory leukocytes elicited by coalmine dust inhalation was increased within 8 days of dust exposure. The increase was sustained for up to 64 days post-exposure. The overall proteinase burden in the alveolar region is related to both total leukocyte number and to the proteolytic activity of the inflammatory leukocytes and is therefore greatly increased (6-fold increase compared with controls) in the coalmine dust animals. Such an increase may lead to levels of proteinase in the alveolar region that are sufficient to overload the antiproteinase screen and permit connective tissue proteolysis to take place.

4.8.4. Inhibition profile of coalmine dust-elicited leukocytes

As previously described with other particles (4.4.), the inhibitor profile of the inflammatory leukocytes elicited by coalmine dust suggested that the proteolytic activity of inflammatory bronchoalveolar leukocytes was mediated by serine proteinases. the serine proteinase inhibitor, α1-PI was a more effective inhibitor of the leukocytes than α2M or the metalloproteinase inhibitor EDTA, even although the leukocyte population was around 97% macrophages. It is unlikely that the 3% neutrophils in the population could account for the increased proteolytic activity of the inflammatory leukocytes. Monocytes secrete serine proteinases (Senior and Campbell, 1983) and thus newly-recruited cells may make a contribution to the serine proteinase activity of the bronchoalveolar leukocytes. However, monocytes change into morphologically typical alveolar macrophages within a few hours of entering the alveolar space (Van oud Alblas et al, 1979) and so the actual levels of serine proteinase released by monocytes may be small.

Further work with different specific inhibitors of serine and metalloproteinases is therefore warranted to identify the type and cellular source of fibronectin-degrading activity in the bronchoalveolar leukocytes.

4.8.5. *In vitro* triggering of the proteolytic activity of coalmine dust-elicited bronchoalveolar leukocytes

In vitro treatment of inflammatory leukocytes with triggers eight days after the start of dusting, again failed to elicit increased proteolytic activity (see 4.4. and 4.5) and may reaffirm the unresponsiveness of alveolar leukocytes. However, in this part of the study the proteolytic activity of the leukocytes increased eight days after the start of dust-exposure but did not increase further thereafter. The proteolytic activity of the dust-elicited leukocytes may therefore have been at maximum before the start of triggering.

4.8.6. Pathogenesis of pneumoconiosis

In the coalmining industry, continuous inhalation exposure to coalmine dust causes the accumulation of elevated numbers of inflammatory leukocytes in the bronchoalveolar region (Begin et al, 1986) and bronchoalveolar leukocytes from coalminers with pneumoconiosis secrete increased levels of neutral proteinases (Hayem et al, 1980). Results from the intratracheal exposure experiments in the present study (4.6.) link the ability to sustain alveolar inflammation and to inflict a prolonged insult of inflammatory leukocyte-derived proteinases on the lung tissue, with the potential to cause pathological change in the lung. The finding of increased levels of leukocyte proteinases in the bronchoalveolar lavage fluid of coalworkers with pneumoconiosis (Sablonniere et al, 1983) firmly implicates such damage in the tissue derangements of fibrosis in coalworkers pneumoconiosis (Ruckley et al, 1984) and may play a major part in the development of emphysema in this disease.

4.8.7. Experimental results related to the progressive nature of pneumoconiosis

Persistence of bronchoalveolar inflammation, with increased proteolytic activity of the inflammatory leukocytes, up to 64 days after cessation of dust exposure, suggests a possible mechanism for the progression of pneumoconiosis in miners long after cessation of dust exposure (Ruckley et al, 1984). It also lends support to one report that pulmonary inflammation persists in retired coalmine workers as measured by clearance of $Tc^{99}m$ DTPA (Susskind et al, 1989). These findings have implications for the long-term health of persons who stop working in environments with high levels of coalmine dust but who may have ongoing inflammation and increased proteinase levels in their lungs.

4.9. Investigation of the pathogenicity of silica in relation to the free quartz surface

4.9.1. Previous work on the surface modification of silica particles

The inflammogenic potential of silica has been related to its surface properties (Kreigseis et al, 1987), which may be modified by compounds occurring in conjunction with quartz in mixed dusts. Such compounds include iron, which reduces the fibrotic potential of silica (Gross et al, 1960; Reichel et al, 1977) and aluminium which reduces both the inflammogenicity and the fibrogenicity of silica (Begin et al, 1987a,b; Le Bouffant et al, 1977a). Aluminium is absorbed onto the surface of silica particles (Denny et al, 1939), rendering it less refractive by electron diffraction, (Le Bouffant et al, 1977b), increasing its zeta potential (Nolan et al, 1981) and increasing flocculation by reducing the solubility of the particles (Denny et al, 1939). In addition, the quartz component of native silica dust can be surface-modified by techniques that remove contaminants from the surface of the quartz particles and render them more toxic to cells *in vitro* (Kreigseis et al, 1987). The importance of the surface reactivity of quartz has also been demonstrated by showing that freshly fractured quartz has greater biological activity - presumably because new, uncontaminated surfaces are revealed (Heffernan, 1932; Fubini et al, 1987). Clay minerals such as kaolin, mica, illite or smectite are potential sources of aluminium in mixed dusts and a pilot study by Le Bouffant et al (1977b) has demonstrated reduced biological activity of quartz when administered in conjunction with illite. Further experimental evidence *in vivo* in support of the "antidotal rocks" theory of Bremner (1939) has demonstrated that treatment with various aluminium or iron compounds in parallel with quartz-dosing markedly reduced the pathogenicity of the quartz in rats (Gross et al, 1960; Le Bouffant et al, 1977a), Guinea pigs (Gross et al, 1960), mice (King et al, 1953) and rabbits (Dworski, 1955). However, these studies reflected only the final outcome of the disease process by measuring the extent of the pathological change in the lungs. In addition, assessment of the protective effect of the aluminium and iron was complicated by the fact that they were particulate in nature and could elicit an adverse reaction in the lung in their own right (Shaver, 1948; Jordan, 1961; Reichel et al, 1977).

4.9.2. Inflammogenicity of aluminium lactate-coated quartz

More recently, experimental studies on the biological activity of quartz in vivo have used soluble aluminium to modulate the effect of quartz in eliciting an inflammatory response and subsequent pathological change in the lungs of sheep (Begin et al, 1987a). These studies have shown that pre-treatment of quartz with soluble aluminium reduces the

inflammatory response as measured by bronchoalveolar lavage; tissue damage was also markedly reduced.

In the present study, pretreatment of quartz with aluminium lactate reduced the total number of leukocytes and percentage of neutrophils recruited to the alveolar region but did not fully reduce the proteolytic activity of the leukocytes. These results are similar to those found in the coal inhalation study where, 64 days post-exposure, the total leukocytes returned to background levels but the percentage neutrophils and the proteolytic activity of the population remained elevated; the overall potential proteinase burden in the bronchoalveolar region (ie protease/cell x total cells in lavage) was 5-fold greater than the controls. Similarly, in the animals dosed with quartz pre-treated with aluminium lactate, total cells in lavage returned to background levels two months after the intratracheal injection. However, although there was a decrease in both the percentage of neutrophils in the population and in the proteolytic activity of the leukocytes, both of these parameters remained greater than controls; the total potential proteinase burden in the bronchoalveolar region in the quartz/aluminium lactate treated animals was 4-fold that of the controls. This indicates that, although the harmful potential of the quartz particles was attenuated by the aluminium treatment, it was not completely abolished. This may be due to incomplete coating of the quartz particles or to other aspects of quartz reactivity which are unaffected by the aluminium treatment.

4.9.3. Effect of delayed treatment with aluminium lactate on established quartz-induced alveolitis

Late dosing with aluminium lactate one month into quartz exposure did not eliminate the existing alveolitis and failed to reduce the proteolytic activity of the bronchoalveolar leukocytes but did reduce the progression of the inflammatory response. This suggested either that the aluminium lactate could gain access to the quartz particles in the lung or that it was acting by suppressing the inflammatory leukocytes. The alveolitis evoked by C.parvum was, however, unaffected by in vivo aluminium lactate treatment which suggests that the effect of the aluminium lactate was due to some interaction with the quartz particles and was not suppressive of the inflammatory leukocytes.

4.9.4. Histological examination of control, quartz and quartz/aluminium lactate treated rat lungs

In paraffin section the tissue response agreed with the progressive nature of the bronchoalveolar lavage results in quartz-exposed rats. Increasing tissue injury culminated in marked alveolar lipoproteinosis and hyperplasia within the alveolar septa at three

months. Reflecting the inflammatory response, the development of tissue changes in response to quartz pre-treated with aluminium lactate was progressive but was markedly attenuated compared with quartz alone. These results are in agreement with Begin et al (1987a) who also demonstrated attenuated leukocyte responses and pathological change with aluminium-coated quartz in sheep lungs. In the present study, late dosing with aluminium lactate after the quartz response was established produced divergent responses - the alveolitis was markedly reduced but there was no effect on the extent of tissue injury; Begin et al (1987b) reported similar results in exposed sheep.

4.9.5. The biological activity of quartz in mixed dusts

The foregoing results strongly suggest that the free quartz surface is important in mediating the pathogenic properties of quartz and that its biological activity is diminished in the presence of aluminium. In mixed dusts, the pathogenic effect of quartz may be similarly modulated by interaction with other minerals in the dust such as iron or aluminium silicate clays. The interaction between quartz and aluminium can occur subsequent to a substantial quartz-induced alveolitis but still significantly reduces the activity of the quartz. Thus, in situations such as coalmining, where there can be significant temporal variation in the mineralogical content of mixed dust, the pathogenic effect due to inhalation of a high quartz content dust might be attenuated by subsequent inhalation of a dust containing ameliorating minerals. These results may have a bearing on assessing the potential harmfulness of any mixed dust but further research is required to understand the nature of the interactions between quartz and other mineral components present in mixed dusts.

4.9.6. Prophylactic treatment with aluminium lactate

There have been many previous studies which have attempted to use aluminium therapy as treatment in pneumoconiosis but the results have been inconclusive (Berry, 1948; Kennedy, 1956; Dix, 1971). The present study suggests reasons for the variability of the effectiveness of aluminium treatment by indicating that such treatment would reduce, but not eliminate the harmful potential of inhaled quartz. Aluminium, however, exerts harmful effects on the body in its own right (Sigel and Sigel, 1988). The use of aluminium treatment in the prophylaxis of pneumoconiosis would only be warranted, therefore, in cases of severe, acute silicosis which might otherwise be fatal within a short period of time. While dust suppression must remain the rational goal in any strategy for reducing pneumoconiosis, human error and ignorance will ensure that high exposure will still occur and in these cases, any rational prophylaxis is worth considering at least.

4.10. Summary

This work has investigated the mechanisms whereby connective tissue proteolysis may lead to the fibrotic lesions of pneumoconiosis and the emphysema which often accompanies them. The main findings of the study are as follows:-

I Particles deposited in the alveolar region of the lung evoke an inflammatory response which is manifest as increases in the total number and percentage of neutrophils in the bronchoalveolar lavage.

II Inflammatory bronchoalveolar macrophages and neutrophils have increased proteolytic activity compared with resident alveolar macrophages and together with the increased number of leukocytes, this leads to a markedly increased proteolytic burden in inflamed lungs.

III Measuring the proteolytic activity of intact leukocytes gives a better indication of their overall ability to damage the extracellular matrix of the alveolar septa than can be obtained by measuring a single secreted enzyme such as elastase.

IV The magnitude of the initial inflammogenic response to particles deposited by intratracheal instillation is not related to their pathogenic potential but the duration of the alveolitis appears to be a major descriptor of their potential harmfulness.

V The ability of particles to evoke and sustain an inflammatory response is not solely reliant on particle durability.

VI The magnitude of the persistent alveolitis in response to quartz and coalmine dust in the lung, in common with the increased proteolytic activity of the bronchoalveolar leukocytes, reflects the pathogenic potential of the two dusts and may be associated with disease progression.

VII The inflammogenic properties of quartz are related to its surface reactivity in biological systems. The ability of aluminium to modify the surface of the quartz particles, so rendering them less harmful, suggests that this may occur in mixed dusts containing aluminium silicate clays. Coating of the quartz particles may account for the low prevalence of pneumoconiosis in some collieries where there is a high quartz content in the coalmine dust.

VIII Bronchoalveolar leukocytes are less readily activated than peripheral blood neutrophils *in vitro*. This may be a reflection of the unique situation of the

bronchoalveolar leukocytes in that they are constantly exposed to exogenous material. Inflammatory responses which would be appropriate elsewhere in the body, in the lungs could quickly lead to life-threatening alveolitis and tissue destruction and are therefore suppressed.

IX A portion of the proteolytic activity of both resident and inflammatory leukocytes is non-inhibitable. In healthy lungs, this may be associated with normal tissue turnover but in inflamed lungs it may be the means whereby proteolytic damage occurs despite the presence of the antiproteinase screen.

X The proteinase burden in inflamed lungs may be derived from either, or both the macrophages and neutrophils which constitute the alveolitis.

This study has investigated the mechanisms whereby inhalation of mineral dusts such as coalmine dust and quartz can lead to the development of pneumoconiosis. The influence of type of particle, duration of exposure, retention of biological reactivity of the particles and how that reactivity can be modified have been considered. The relationship between the alveolitis and the disease process has been addressed by assessing the potential role and the type of leukocyte proteinases involved in the disease process. The outcome of the study is to provide some insight into the way that disease develops in relation to dust exposure. The results may be of use in developing treatment for men who have already been exposed to harmful levels of dust but may also help in developing effective control strategies in order to prevent such exposures in the future.

4.11. Further work arising from the study

1 Knowledge of the role of macrophage and neutrophil proteinases could be of importance in the development of inhibitors of leukocyte proteinases for use in the alveolar region. A considerable amount of research effort has been expended in developing inhibitors of neutrophil elastase but there has been little work done in the field of macrophage proteinases. An investigation of the type and inhibitability of inflammatory macrophage proteinase would be useful following the demonstration in this study that inflammatory macrophages have considerable proteolytic activity and may play a part in tissue damage in chronic inflammatory lung disease. Such a study could be accomplished, initially, by further use of specific proteinase inhibitors in the fibronectin proteolysis assay.

2 Inflammatory mediators are likely to be present in the milieu of dust-inflamed lungs and may account for the increased proteolytic activity of the lavaged leukocytes. The non-responsiveness of resident alveolar macrophages to dust exposure *in vitro*

makes them an ideal model for investigating the role of cytokines such as IL-1, TNF and gamma-interferon in increasing the proteolytic activity of macrophages in dust-exposed lungs. The question could be addressed by treating control macrophages *in vitro* with a variety of particles of known pathogenic potential and measuring their proteolytic activity as above. Recombinant cytokines could then be added into the assay, either alone or in combination to assess the effects of synergy.

3 The *in vivo* exposure model would lend itself to assessing the effect of drugs in modulating the inflammatory response in the bronchoalveolar region.

4 All of the experiments in the present study have been carried out using a single dose of particles. Further work to establish dose-response curves for the particles, particularly quartz, should be carried out.

5 Estimation of the persistent inflammogenic potential of coalmine-dust was carried out over a very short time. It would be very interesting to extend the time period to determine whether the inflammation would resolve. Results not presented in this thesis, have indicated that the alveolitis in response to a single intratracheal dose of 1mg of quartz, persists for at least one year. The magnitude and duration of the alveolitis in response to smaller amounts of quartz are also of interest.

6 The leukocyte populations tested in this study were obtained from the bronchoalveolar space as a first step in understanding the mechanisms of the pathogenesis of chronic inflammatory lung disease in general and pneumoconiosis in particular. The next phase of the work should encompass a study of the interstitial leukocytes. These are the cells that are in the most intimate contact with the fixed cells and connective tissue of the alveolar septa and may therefore be more important to pathological change than the alveolar macrophages.

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